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Molecular characterisation of the bacterial communities in cabbage aphid (Brevicoryne brassicae) and their associated fitness effects

Clark, Emily

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Molecular characterisation of the bacterial communities in cabbage aphid (*Brevicoryne brassicae*) and their associated fitness effects

Emily Louise Clark

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APSE - bacteriophage, *Acyrthosiphon pisum* secondary endosymbiont
BHQ - Black Hole Quencher®
BLAST - basic local alignment search tool
bp - base pairs
BSA - bovine serum albumin
BWYV - beet western yellows virus
CaMV - cauliflower mosaic virus
DAPI - 4'-6-diamidino-2-phenylindole
DGGE - denaturing gradient gel electrophoresis
DNA - deoxyribonucleic acid
dNTPs - deoxyribonucleotide triphosphates
EDTA - ethylenediaminetetraacetic acid
FAM - 6-carboxyfluorescin
FISH - fluorescent *in situ* hybridisation
FRET - fluorescence resonance energy transfer
g - G-force
g - gramme
IGS - inter genic spacer
IPTG - isopropyl β-D-1-thiogalactopyranoside
ITS - trans genic spacer
Jak/STAT - janus kinase/signal transducers and activators of transcription proteins
kbp - kilo base pairs
l - litre
ml - millilitre
mg - milligramme
mM - millimolar
MBG - molecular biology grade
NCBI - National Center for Biotechnology Information
PAXS - pea aphid X-type symbiont
PCR - polymerase chain reaction
PGRPs - peptidoglycan recognition proteins
POA - partial order alignment
qPCR - quantitative polymerase chain reaction
RAxML - randomised axelerated maximum likelihood
RQ - relative quantity
rDNA - ribosomal deoxyribonucleic acid
RNA - ribonucleic acid
ROX - carboxy-X-rhodamine
SDS - sodium dodecyl sulphate
SOPE - *Sitophilus oryzae* primary endosymbiont
SZPE - *Sitophilus zeamais* primary endosymbiont
T-RF - terminal restriction fragment
T-RFLP - terminal restriction fragment length polymorphism
Tris - tris (hydroxymethyl) aminomethane
TuMV - turnip mosaic virus
T3SSs - type 3 secretion systems
µg - microgramme
µl - microlitre
µm - micrometre
µM - micromolar

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Declaration

Student Declaration

I hereby declare that the following thesis is based on the results of investigation conducted by myself, and that this thesis is my own composition. This thesis has not in whole or any part, been previously presented for a higher degree. Work other than my own is clearly stated in the text with reference to the relevant researchers or their publications.

Emily L. Clark

Supervisor Statement

The relevant Ordinance and Regulations have been fulfilled by the candidate.

Professor Stephen Hubbard

Abstract

Aphids harbour symbiotic bacteria that can have positive, negative or neutral effects on their survival and performance. These bacteria are split into two groups: the primary obligate endosymbiont, *Buchnera aphidicola*, and the secondary 'facultative' bacteria. In pea aphid (*Acyrthosiphon pisum*) three vertically transmitted secondary facultative bacteria have been shown to influence various fitness traits in their aphid hosts, including susceptibility to natural enemies. Very little is known however, about the fitness effects of bacterial associations in other aphid species. The aim of this study was to characterise bacterial diversity in a Scottish arable pest, the cabbage aphid (*Brevicoryne brassicae*), and investigate whether bacterial composition influences trophic interactions by testing their impact on aphid–parasitoid interactions.

The bacterial community of cabbage aphid was quite different to pea aphid, both in terms of the density of the primary symbiont, *Buchnera*, but also in secondary bacterial complement. There was a wide diversity of bacteria associated with the cabbage aphid although these were not the three commonly studied pea aphid secondary symbionts and were likely to be a different type of symbiont, relying primarily on horizontal transmission. Phylogenetic analysis of 16S sequence revealed that the majority of bacterial types could be split into two groups: Group 1 *Pseudomonas* type bacteria and Group 2 *Erwinia* type bacteria. A real-time (Taqman®) qPCR assay was used to determine the infection status of the cabbage aphid lines and indicated there were four different community types in cabbage aphid: (1) aphid lines dominated by Group 1 bacteria; (2) aphid lines dominated by Group 2 bacteria; (3) aphid lines that harboured large approximately equal amounts of each group of bacteria; (4) aphid lines that harboured relatively little of either group of bacteria.

The molecular results provided a basis for aphid–parasitoid fitness experiments. Preliminary results, based on single aphid genotypes, indicated that harbouring Group 2 *Erwinia* bacteria had both direct and indirect fitness costs by reducing cabbage aphid fitness and positively influencing the fitness of emergent *Diaeretiella rapae* parasitoids. The extent to which bacterial associations can influence aphid fitness and the truly multitrophic nature of cabbage aphid population dynamics in arable systems are highlighted by this study.

1. General Introduction

1.1 Aphids as Successful Crop Pests

1.1.1 The cabbage aphid.

Aphids belong to the superfamily *Aphidoidea*, which is an extremely successful insect group. Soft bodies, membranous wings and a diet comprised entirely of plant sap characterise the aphids (Dixon 1973; Dixon 1998). There are approximately 4400 species of aphid worldwide with numerous host plant associations (Blackman and Eastop 2000). The adaptive radiation of aphids into the aphid species–host plant associations we see today is thought to have occurred with the appearance of flowering plants (Angiosperms), although aphids actually originated earlier, in the Permian era probably around 280 million years ago (Moran *et al.* 1999). The host plants of most present-day aphids are Angiosperms, many of which are crop plants. Numerous species of aphid are agricultural pests that can cause significant damage to crops resulting in large economic losses. For example, in Scotland where a large number of brassica crops are grown, the cabbage aphid (*Brevicoryne brassicae*) (Linnaeus) (Hemiptera: Aphididae: Aphidinae: Macrosphini) is a particular problem.

Cabbage aphids (*B. brassicae*) (Plate 1.1) are small (2–2.5 mm long) and covered with a greyish waxy layer. They form dense aggregations (Plate 1.1) on the leaves, petioles and developing infloresences and vegetative branches of brassica plants that become established in July and are present until late October. Cabbage aphids differ from other aphid species by feeding obligately on crucifers which are high in plant secondary metabolites called glucosinolates. When attacked the plant glucosinolates are hydrolysed by the enzyme myrosinase to yield toxic compounds such as isothiocyanates, thiocyanates and nitriles (Bones and Rossiter 1996). Cabbage aphids are able to store glucosinolates and have independently evolved their own myrosinase capable of hydrolysing a number of glucosinolates including sinigrin (Jones *et al.* 2002; Bridges *et al.* 2002). When the aphid body is damaged, the insect myrosinase releases toxic isothiocyanates by glucosinolate hydrolysis as a defence against natural enemies (Francis *et al.* 2001; Kanzana *et al.* 2007).

Plate 1.1: An aggregation of cabbage aphid nymphs and adults on a brassica leaf.

1.1.2 Aphid damage to crops.

All aphid species, including cabbage aphids, feed on phloem sap, which they obtain by tapping into the phloem with their stylets (Dixon 1998). Phloem sap flows under pressure and is forced up the stylet when aphids access the phloem vessels (Dixon 1973; Dixon 1998). Aphids preferentially feed on younger plants, which have a higher nutritional quality with larger concentrations of sugars, non-essential amino acids and soluble nitrogen, so often crops are affected in the early stages of development (Dixon 1998; Douglas 2003). Aphids cause direct and indirect feeding damage to crops. Aphid feeding causes direct mechanical damage, which makes the plant stunted, yellow and distorted with malformation of new growth and curled leaves. Mechanical damage results from diversion of plant resources away from the growing parts of the plant to the aphid feeding sites (reviewed in Goggin 2007). These changes in host physiology are beneficial to the aphid, providing a nutrient source and safe sheltered environment in curled leaves, but are highly deleterious to the quality and yield of the host plant.

Aphids can also cause damage to crops indirectly through plant virus transmission. Viruses are acquired as the aphid probes the phloem, and are transferred via the saliva when the aphid probes other plants (Perring *et al.* 1999). Aphid transmitted viruses either kill the infected plant or reduce crop yield and quality to the point that it is not worth harvesting (Eastop 1977)**.** Oilseed rape (*Brassica napus*) and the other brassica crops grown in Scotland are susceptible to a number of different viruses including cauliflower mosaic virus (CaMV), turnip mosaic virus (TuMV), broccoli necrotic virus (BNYV) and beet western yellows virus (BWYV) (Broadbent 1958; Walsh and Tomlinson 1985; Hardwick *et al.* 1994; Raybould *et al.* 1999; Rimmer *et al.* 2007). Cabbage aphid is one of approximately 27 aphid species that are known vectors of CaMV (Namba and Sylvester 1981*;* Markham *et al.* 1987; Palacios *et al.* 2002; Moreno *et al.* 2005) and it can also transmit TuMV virus but not BWYV (Smith and Hinckes 1985; Herrbach 1994). Hardwick *et al.* (1994) investigated TuMV, CaMV and BWYV in oil seed rape in the UK from 1991–1993 and reported a reduction in yield of approximately 70–79% in oilseed rape plants showing severe virus symptoms. They found that on average less than a third of a crop was infected by any of the three viruses at any one time, which is sufficient to reduce income generated from the crop.

Brassica crops contribute significantly to the Scottish agricultural economy (Table 1.1). Methods to control aphid populations to protect crops from aphid borne viruses and mechanical damage include ploughing fields directly after harvesting (destroying the eggs of over-wintering aphids), rotating with non-host crops for the problem aphid, application of pesticides and biological control with parasitoid wasps and other natural enemies (Blackman and Eastop 2000).

Table 1.1: Example of brassica crop production in Scotland (figures according to Scottish Government 2007 census http://www.scotland.gov.uk)

Brassica Crop	Crop Type	Land Covered (Hectares)	Value (million £)	% of Total Area for Crop Type
Oilseed Rape (*Brassica napus*)	Combinable	36,571	31	8
Turnips (*Brassica rapa*) and Swedes (*Brassica napobrassica*)	Horticultural	1773	21	22
Brussels Sprout (*Brassica oleracea* var. *gemmifera*)	Horticultural	820	14	15
Kale (*Brassica oleracea* var. *acephala*) and Cabbage (*Brassica oleracea* var. *capitata*)	Fodder	2887	Unknown	12

1.1.3 The life cycle of aphids.

Aphid populations are difficult to control due to their unique life history pattern, which allows rapid growth of populations in a short period of time. The life cycle of the *Aphidoidea* underpins their evolutionary success as an insect group. Different species of aphid undergo different life cycles depending largely on prevailing environmental conditions. Many aphid species exhibit holocycly and in the warmer periods of the year when the day length is longer aphid populations comprise parthenogenetic females that do not require fertilisation and are viviparous (Dixon 1973; 1987; 1998). The eggs of the parthenogenetic female begin development within the aphid immediately after ovulation so an aphid nymph can have embryos developing within its body that also have embryos, known as the 'telescoping of generations' (Dixon 1973; 1998). 'Telescoping' shortens the period between generations by reducing the length of time between the final moult and the onset of reproduction, allowing rapid rates of population increase (Dixon 1987). Adult aphids can be either alate (winged) or apterous (wingless) morphs; the winged aphids can disperse and the wingless aphids remain on the parent plant and reproduce (Dixon 1998).

In a holocyclic life cycle the periods of asexual parthenogenetic reproduction in spring and summer are interspersed with periods of sexual reproduction when the weather is cooler in autumn and winter. Photoperiod and temperature are thought to be the trigger for aphids to switch from asexual to sexual reproduction. The majority of aphid species are autoecious, surviving on a single host throughout their life cycle while around 10% of aphid species alternate between a primary host plant upon which they over-winter, and a secondary host plant upon which they spend the summer months (Moran 1992). Sexual reproduction occurs on the primary host plant. In the latter case specialised winged migrants, known as gynoparae, are produced in the autumn and fly from the secondary host to the primary host to deposit sexual morphs. In the sexual phase, male sexual morphs mate with oviparous females, which produce eggs. Once the eggs have been laid the adult aphids die and the population over-winters as eggs. The following spring when plant growth resumes, viviparous winged females hatch from the eggs, giving rise to a series of parthenogenetic generations and rapid population expansion on summer host plants (Dixon 1973; 1987; 1998). Once viviparous winged

females hatch they can use wind aided flight to cross relatively large geographic ranges and colonise new areas (Robert 1987).

Holocycly is exhibited by a number of aphid species including the Blackberry–cereal aphid (*Sitobion fragariae*) (Walker) (Hemiptera: Aphididae: Aphidinae: Macrosiphini) which alternates between the primary host blackberry (*Rubus fruticosus* agg.) and the Poaceae (grasses, mainly cereals) (Hand 1989). The cabbage aphid also exhibits a holocyclic life cycle but is confined to the Brassicaceae and over winters as black eggs on petioles and folds and in host plant debris near the soil surface (Blackman and Eastop 2000). In contrast some aphid species such as the giant willow aphid (*Tuberolachnus salignus*) (Gmelin) (Hemiptera: Aphididae: Lachninae: Lachnini) exhibit an obligate asexual 'anholocyclic' life cycle during which they only produce parthenogenetic females. Although obligate asexual reproduction allows aphid populations to remain active year round it also leaves them vulnerable if environmental conditions deteriorate. As a consequence some aphid species practice 'bet hedging'. The bird cherry–oat aphid (*Rhopalosiphum padi*) (Linneaus) (Hemiptera: Aphididae: Aphidinae: Aphidini), for example, alternates between reproductive strategies according to environmental conditions, i.e. if temperature and day length fall below a threshold level it reproduces sexually. Outwith these conditions it reproduces by parthenogenesis and consequently within the period of a year *R. padi* might not reproduce sexually at all (Hand 1989).

1.1.4 Factors controlling aphid population dynamics.

Although most aphid species in temperate regions are cyclically parthenogenetic (holocyclic), there is some evidence to suggest that during milder winters aphid species, such as cabbage aphid, are able to over-winter as parthenogenetic adults by remaining on the stems of harvested crops or on alternative host plants (anholocycly). The milder winters that have been predicted as a consequence of environmental change are therefore likely to have a profound influence on aphid population dynamics (Mondor *et al.* 2005). If aphids over-winter as adults they are able to commence asexual parthenogenetic reproduction earlier in the growing season. This could particularly affect the productivity of autumn sown crops. In the UK, oilseed rape is largely spring sown (March/April) with some winter sown crops (August/September). Other food and

fodder brassica crops such as broccoli (*Brassica oleracea* var. *italica*) and cabbage (*Brassica oleracea* var. *capitata*) are grown and harvested through the winter in the UK. The method of brassica farming in the UK therefore provides access to host plant material for brassica pests such as cabbage aphid for most of the year as there is only a short break between harvesting the spring crop and sowing the winter crop (Walsh and Tomlinson 1985). As a consequence, cabbage aphids could remain active in the crop even over the winter months, allowing rapid population development in the spring on emerged autumn sown oilseed rape (Shahraeen *et al.* 2003).

Predicting how aphid populations will react to environmental change is difficult because we still know relatively little about the multiple factors that can influence aphid fitness in natural populations. Although aphid populations increase rapidly they do not multiply to the extent that would be expected given their life history strategy. Instead they are constrained by a number of abiotic and biotic factors including climatic variation (Mondor *et al.* 2005), pressure from natural enemies (Hufbauer 2002) and host plant quality (Karley *et al.* 2004; Powell *et al.* 2006; Karley *et al.* 2008). Therefore, strong selection pressures imposed by these environmental variables are the driving forces shaping the ecological and evolutionary dynamics of aphid populations (Gwynn *et al.* 2005). Additionally, interactions between aphids and plant viruses, natural enemies and bacteria are all known to influence the fitness of aphid populations (reviewed in Karley *et al.* 2004 and Goggin 2007). Identifying the factors that determine aphid fitness is fundamental to understanding the seasonal dynamics of aphids in crop systems and optimising the methods that are used to control aphids such as pesticide application or biocontrol strategies using aphid natural enemies.

1.2 Parasitoid Biology and Their Role as Aphid Natural Enemies.

1.2.1 Parasitoid life history.

Parasitic hymenopteran wasps are a large and diverse group of insects that are prevalent throughout all of the United Kingdom and the rest of the world except the polar regions. In the UK alone there are approximately 6500 species of hymenoptera, the majority of which are parasitoids (Quicke 1997). An insect parasitoid is defined as an insect whose larval stage feeds on the body of another insect or spider (Godfray

1994). Aphids are hosts to three groups of hymenopteran endoparasitoids, although the majority of the 400 species that parasitise aphids are in the families Braconidae (Aphidiinae) and Aphelinidae (Godfray 1994; Mackauer *et al.* 1996). Aphidiid wasps represent one of the best studied groups of parasitoid wasps because members of the group, such as *Aphidius ervi* (Haliday) (Hymenoptera: Braconidae: Aphidiinae), are commonly used for the biological control of aphids. All aphid parasitoids are termed endoparasitoids because they lay their eggs within the body of the host insect (Godfray 1994). This method of placing the eggs directly where there is a nutrient rich food source has been a key feature in the evolution of parasitoids and the primary driver for their success as an insect group (Quicke 1997). The female parasitoid uses a variety of olfactory, chemosensory and visual cues to locate suitable aphid hosts (reviewed in Mackauer *et al.* 1996). Once she has located a suitable host she injects her eggs into the aphid using a highly specialised ovipositor that stings the host and temporarily paralyses it (Godfray 1994). The resulting larva feeds and develops within the aphid's body for a period of approximately two weeks, depending on species and environmental variables, and the aphid host eventually dies. Aphid parasitoids are known as koinobiont because they allow the aphid to grow in size after it has become parasitised (Godfray 1994). Koinobiont parasitoids develop through four larval instars within the host, during which the host can continue to grow until the larva feeds on its vital organs, usually during the final stages of development (Godfray 1994; Quicke 1997). As the aphid is consumed the developing larva cements the remaining outer coat of the aphid body to the surface of the plant and develops within the protective shell, which is commonly referred to as a 'mummy' (Godfray 1994). After pupation, the parasitoid wasp emerges from the mummy as a reproductive adult (Godfray 1994).

Successful parasitism is a very complex achievement as the parasitoid must locate and assess a suitable host then overcome the host's natural defences against parasitism. Once within the host the developing larva has to adapt to or regulate the constantly changing environment within the aphid body to satisfy its own metabolic, nutritional, and ecological needs (Falabella *et al.* 2000; Brodeur and Boivin 2004). Consequently, the association between the aphid and the parasitoid larva is tightly conserved both chemically and physiologically and can be influenced by a number of abiotic and biotic factors. Recent research has focussed on the effects of temperature

(Campbell *et al.* 1974; Li and Mills 2004; Hance *et al.* 2007), aphid natural defences (Kanzana *et al.* 2007), host specificity (Blande *et al.* 2004; Antolin *et al.* 2006; Bayhan *et al.* 2007), plant volatiles (Girling *et al.* 2006), aphid genotype (Ferrari *et al.* 2001), aphid developmental stage (Martinou and Wright 2007) and the bacterial complement of the host aphid (Ferrari *et al.* 2004; reviewed in Oliver *et al.* 2010). Several mathematical models have been built for predicting the dynamics of parasitoid populations that incorporate a number of the above variables (Mondor and Roitberg 2000; Schofield *et al.* 2002; Rauch and Weisser 2007; Stark and Acheampong 2007). Understanding the biology of parasitoid wasps and the many factors that could influence the aphid–parasitoid interaction is essential to ensure that such models include relevant influencing factors.

1.2.2 Parasitoids as biological control agents of aphids.

Biological control is a method of controlling agricultural pests using their natural enemies rather than pesticides. Parasitoid wasps are of immense importance in both natural and agricultural systems because they influence the population density of many crop pests including aphids (Hufbauer 2002). There are examples of parasitoid wasp species that have been used effectively for the biocontrol of aphids (Milne 1999; Beckage 2004; Levie *et al.* 2005). The koinobiont parasitoid *Diaeretiella rapae* (McIntosh) (Hymenoptera: Braconidae: Aphidiinae) shows a strong attraction to semiochemicals from crucifer plants (Vaughn *et al.* 1996) and commonly attacks *B. brassicae* (Plate 1.2). Consequently, *D. rapae* has been suggested as a possible biocontrol agent for cabbage aphid (Zhang and Hassan 2003). Although, natural enemies do not guarantee effective protection and cannot completely control host populations they can offer economic and human health savings by providing an alternative to pesticides.

Finding the correct parasitoid species to use for biocontrol has however often proved difficult. Generalist parasitoids that attack a range of aphid species are often only effective at controlling a few species, therefore selecting the correct parasitoid–aphid species combination is essential for aphid control. For example, although there are approximately sixty aphid species that are potential hosts for *D. rapae*, only five or six aphid species are commonly attacked (Némec and Stary´ 1994; Pike *et al.* 1999). In

addition, parasitoid wasps are particularly sensitive to population effects. In some areas where parasitoids are endemic they and their hosts do not exist at high population densities (Hughes 1963; Costello and Altieri 1995; Nieto *et al.* 2006; Bahlai *et al.* 2010; Noma *et al.* 2010). The patchy fragmented distribution of aphid populations constrains parasitoid wasp populations (Pareja *et al.* 2008) leaving them vulnerable to small population effects, such as reproductive isolation and 'founder' effect that lower genetic variability and increase the risk of extinction (LaSalle and Gauld 1991; Weisser 2000; Fathipour *et al.* 2006; Rauch and Weisser 2007). Consequently, when parasitoid wasps are applied as biocontrol agents in open vegetation, they seldom establish in the wild population and thus only provide a temporary solution to aphid infestations.

Extinction threats are of particular concern since the nature of parasitic wasps also indicates that they may include several keystone species upon which ecosystems depend (LaSalle and Gauld 1991; Bukovinszky *et al.* 2008). For example, a species of parasitoid wasp might suppress aphid numbers allowing native vegetation to flourish and thus prevent invasion by weed species (Schmidt *et al.* 2003). Consequently, parasitoid wasps are likely to have very important roles in maintaining stable community dynamics. Modern farming methods frequently lead to a reduction in field margin and hedgerow habitat and, together with widespread pesticide application, this might reduce parasitoid numbers. The application of agricultural adjuvants in conjunction with aphidicides has been shown to have negative effects on the demographics of parasitoid populations (Acheampong and Stark 2004). A decrease in parasitoid induced aphid mortality could lead to increases in mechanical damage to crops and in the spread of aphid-vectored plant viruses. It is also important to consider that with each extinction of a parasitoid wasp species a potential biocontrol agent could also be lost (Quicke 1997). Aphid parasitoids are therefore intrinsically linked to the health of farmed and native vegetation, and research in recent years has focused on understanding the factors that determine parasitoid fitness and parasitism efficiency.

A

B

***Plate 1.2: (A) An individual* D. rapae *parasitoid on a brassica leaf and (B)* D. rapae *emerging from a* B. brassicae *mummy*.**

1.2.3 Abiotic and biotic factors influencing the aphid–parasitoid interaction.

Abiotic and biotic factors imposed by the environment are key in determining aphid and parasitoid fitness. Temperature acts directly on the physiology of the parasitoid and influences its virulence, including the sex ratio and size of offspring and the development time of the parasitoid larvae (King 1987; Godfray 1994). Aphid populations can also be influenced by temperature, which can limit body size and reproductive output, thus determining the quality and number of available hosts for the parasitoid (Li and Mills 2004). In general, development thresholds for parasitoid wasps are higher than for aphids (Campbell *et al.* 1974). Thus, in spring the aphid population increase initiates before parasitoids start to emerge, allowing establishment of aphid populations (Campbell *et al.* 1974). Temperature determines when the parasitoid wasps emerge from their winter diapause, which begins in the late autumn when the temperature drops and the days become shorter (Polgar and Hardie 2000). Warmer temperatures earlier in the year could move parasitoid emergence forward although they will still remain constrained by the dynamics of their host populations (Hance *et al.* 2007), which could also be active earlier in the year (Mondor *et al.* 2005), potentially upsetting delicate balances. The close association between insects and the environment in which they live is essential for their survival. Temperature requirements vary in different geographic locations even within the same species, indicating adaptation to the local thermal environment (Campbell *et al.* 1974). While local adaptation may be essential for a species to become established, it could also limit its ability to achieve satisfactory host control either early in the season or in novel environmental conditions (Campbell *et al.* 1974). Many of the parasitoid wasp populations used for biological control are introduced to their release area from entirely different geographic regions with mixed success, which is likely to relate to pre-adaptation to the environmental characteristics of their 'native' location. Consequently, it is important to consider temperature as a limiting environmental factor that affects the fitness of parasitoids and thus their impact on aphid populations.

Plant effects are also an important aspect of the aphid-parasitoid interaction (Ferrari *et al.* 2001). The pea aphid in North America has formed several genetically distinct 'host races' that are defined by the plant types upon which each feeds (Henter and Via 1995). Parasitism rate varies between host races, potentially resulting in the

parasitoid favouring particular host races with eventual specialisation and even speciation (Henter 1995; Henter and Via 1995). The extent to which these associations are influenced by the characteristics of the aphid, the plant or both is, however, unclear and they are likely to be associated with fitness trade-offs for both parasitoid and aphid. Plant effects can occur at the plant location stage (Bradburne and Mithen 2000), during the search for the aphid (Blande *et al.* 2004), and during the development of the parasitoid larva (Kanzana *et al.* 2007). Variation in plant chemistry mediates not only interactions between plants and aphids but aphids and their natural enemies (Kanzana *et al.* 2007; Härri *et al.* 2009; Kissen *et al.* 2009; Newton *et al.* 2009).

Certain plant species appear to influence parasitism rate. For example, parasitism rates were twice as high for *A. ervi* when pea aphid, its host, was feeding on red clover as opposed to alfalfa (Hufbauer 2002), and *D. rapae* exhibits varying levels of parasitism of cabbage aphid according to brassica type (Bayhan *et al.* 2007). These differences are often attributed to the chemicals produced by the host plant as a defence against herbivory. Parasitoids are attracted to plant volatiles released in response to aphid feeding which provide the parasitoid with highly specific information about host availability (Mackauer *et al.* 1996; Girling *et al.* 2006). For example, on one plant species *A. ervi* could discriminate between volatiles induced by its host aphid species and volatiles induced by a non-host aphid species (Guerrieri *et al.* 1999). As a consequence, some parasitoid wasp populations could become specialised to a particular plant or aphid species and perform poorly on 'novel' hosts (Antolin *et al.* 2006). *D. rapae* parasitoids have a higher productivity and survival when attacking the host aphid species upon which they had been reared for several generations compared to 'novel' aphid hosts (Antolin *et al.* 2006). Molecular analysis revealed little evidence for host-associated races of *D. rapae*, indicating host preference has not resulted in genetic divergence of parasitoid populations across its range (Baer *et al.* 2004). Specialisation could relate to particular chemical or nutritional traits produced by a particular aphid–plant combination. *D. rapae*, for example, is strongly attracted to the semiochemicals of brassica plants induced by cabbage aphid feeding (Bradburne and Mithen 2000). The characteristics of the plant are therefore important in determining the outcome of aphid–natural enemy interactions and can lead to highly specialised associations.

In addition to the abiotic and biotic and factors influencing the aphid–parasitoid interaction, associations with aphid endobacteria are key in shaping aphid–parasitoid interactions, by influencing both aphid–plant associations (e.g. Tsuchida *et al.* 2004) and aphid resistance to hymenopteran parasitism (reviewed in Oliver and Moran (2009) and Oliver *et al.* (2010)).

1.3 The Symbiotic Bacteria of Aphids

1.3.1 Symbiosis in aphids and other insects. **(This section is reproduced from the author's published work Clark *et al.* (2010))**

Throughout their evolutionary history insects have formed multiple relationships with bacteria. Although many of these bacteria are pathogenic, with deleterious effects on the fitness of infected insects, there are also numerous examples of symbiotic associations. The first usage of the term symbiosis was by Anton de Bary (1879) who referred to symbiosis as "the living together of dissimilarly named organisms". Symbiosis is similarly broadly defined today as "the living together of different organisms, usually in close association with one another, to the benefit of at least one of them" (e.g. Thain and Hickman 2001). Symbiotic bacteria that form obligate or facultative associations with insects and that are located intracellularly in the host insect are known as endosymbionts. Endosymbiosis can be a strong driving force for evolution when the acquisition and maintenance of a microorganism by the insect host results in the formation of novel structures or changes in physiology and metabolism. Symbiotic bacteria are key players in insect–plant interactions influencing many aspects of insect ecology and playing a key role in shaping the diversification of many insect groups (reviewed in Janson *et al.* 2008). A large number of herbivorous insects harbour symbiotic bacteria (Fig. 1.1) and as a consequence are able to thrive on nutrient-poor plant tissues. The gut symbiotic bacteria and protists of wood-feeding insects, for example, play a role in enzymatic degradation of cellulose (Tokuda and Watanabe 2007; Carpenter *et al.* 2010), and the intracellular symbiont *Buchnera aphidicola* (hereafter *Buchnera*) synthesizes essential amino acids for its aphid host (Douglas 1998; Gündüz and Douglas 2009). In addition, symbiotic bacteria can influence the fitness of insects in more subtle ways by, for example, increasing resistance to hymenopteran

parasitism (reviewed in Oliver and Moran 2009 and Oliver *et al.* 2010), increasing thermal tolerance (Montllor *et al.* 2002) and influencing host plant specificity (Tsuchida *et al.* 2004; Ferrari *et al.* 2007). The origin of the association between some endosymbionts and their hosts can be traced back to changes in the life history of the host. The weevil species *Sitophilus linearis* (Coleoptera; Curculionidae), for example, is the only aposymbiotic (= without symbionts) species in the Dryophthoridae weevil family and its lack of endosymbiotic bacteria can be associated with a switch from feeding on the seed of monocotyledons such as cereals, to the more nutritionally balanced seed of the tamarind (Delobel and Grenier 1993). Co-diversification of insects and endosymbiotic bacteria into novel niches highlights the extent to which endosymbiont bacteria can be extremely influential in shaping insect ecology (reviewed in Janson *et al.* 2008).

The symbiont bacteria of insects are commonly divided into two categories. The first category includes obligate endosymbionts, often referred to as primary endosymbionts. These are located in the cytoplasm of hypertrophied cells specialised for endosymbiosis called mycetocytes (also referred to as bacteriocytes) which reside in a specialised organ called a mycetome (also referred to as a bacteriome) (McLean and Houk 1973; Buchner 1965). The most familiar example of a primary endosymbiont is *Buchnera*, the primary symbiont of aphids, although there are many other examples of primary symbioses in herbivorous insects including *Sulcia muelleri* in sharpshooters (Hemiptera; Cicadellidae) and *Carsonella ruddii* in psyllids (Hemiptera; Psyllidae) (Fig. 1.1). The primary symbioses of insects are ancient, 160–180 million years in the case of *Buchnera* (Munson *et al.* 1991; Moran *et al.* 1993), and over evolutionary time the aphid and bacteria have become completely dependent on each other. In return for a stable niche and provision of nutrients the primary symbionts play a functional role in the physiology of the host by synthesising essential nutrients which are missing from the diet of the host insect (Douglas 1998; McCutcheon *et al.* 2009; Douglas 2009; Gündüz and Douglas 2009).

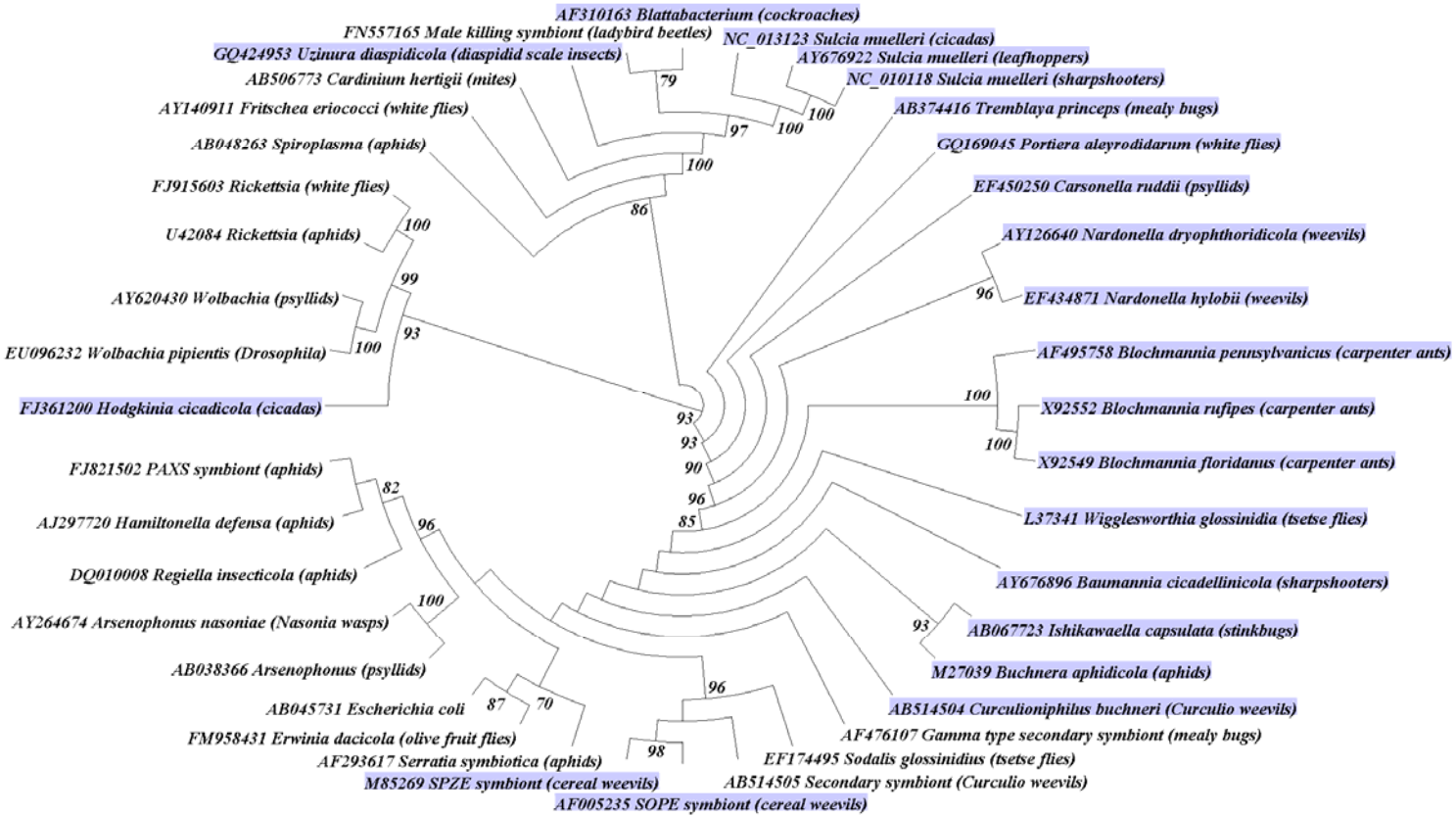


Figure 1.1: Maximum likelihood phylogeny based on available database sequences for the 16S gene to illustrate the diversity of insect symbionts and the major bacterial classes in which they are found. Primary obligate endosymbionts are highlighted in blue. Sequences are preceded by their accession number in the NCBI database. **(This figure is reproduced from the author's published work Clark *et al.* (2010)).**

1.3.1.1 The primary symbiont of aphids **Buchnera aphidicola.**

Buchnera is a γ-3 Proteobacteria allied to the Enterobacteriaceae family of bacteria, which also includes *Escherichia coli* (Munson *et al.* 1991). Almost all members of the *Aphidoidea* possess *Buchnera*, the exceptions being the families Phylloxeridae, which lack symbiotic bacteria, the Adelgidae, which harbour symbiotic bacteria that are morphologically distinct from *Buchnera*, and some members of the Hormaphididae, that form a symbiosis with yeasts (Buchner 1965; Munson *et al.* 1991; Fukatsu and Ishikawa 1996). *Buchnera* is a coccid bacterium 2–4μm in diameter varying in size between aphid species (Mira and Moran 2002), with a thin Gram-negative cell wall (Houk and Griffiths 1980). It is found in the haemocoel of the aphid residing in the cytoplasm of hypertrophied cells called 'primary mycetocytes' (also referred to as bacteriocytes) (McLean and Houk 1973; Buchner 1965; Douglas 1998; Fukatsu *et al.* 2000). There are approximately 60–100 mycetocytes distributed throughout the haemocoel of each single adult pea aphid (Wilkinson and Douglas 1998). The mycetocytes rarely divide but they grow in size as the bacteria within them proliferate, particularly during the period of larval development when the rate of bacterial division is high (Whitehead and Douglas 1993; Baumann and Baumann 1994). *Buchnera* constitute greater than 90% of microbial cells in aphids and contribute 10% of the aphid's total biomass (Douglas and Prosser 1992; Baumann and Baumann 1994). *Buchnera* is transmitted transovarially from one generation to the next either to the egg or to the parthenogenetic embryo (Buchner 1965; Hinde 1971). In oviparious aphids each ovum endocytoses the symbionts forming a 'symbiont ball', while in viviparous aphids the bacteria pass through a pore that appears in the blastoderm (Miura *et al.* 2003; Wilkinson *et al.* 2003). Symbionts gain entry to embryo prior to gastrulation and are subsequently endocytosed by the differentiating mycetocytes (Miura *et al.* 2003; Wilkinson *et al.* 2003). Only a small proportion of the *Buchnera* population, originating from a single maternal mycetocyte, are transmitted from the mother to the offspring resulting in a population bottleneck (Mira and Moran 2002; Miura *et al.* 2003; Wilkinson *et al.* 2003). The effect of repeated population bottlenecks and no opportunity for recombination leaves vertically transmitted primary symbionts such as *Buchnera* vulnerable to population genetic effects such as 'Muller's Rachet' leading to wide scale gene erosion and the build up of deleterious mutations (Moran 2003).

The symbiosis between the aphid and *Buchnera* is considered 'obligate' for two reasons. Firstly, in the absence of *Buchnera* development of nymphs and reproductive output of adults are both reduced (McLean and Houk 1973; Douglas 1989; Ishikawa 1989; Douglas 1992; Douglas 1996; Douglas 1998). Secondly, the genome of the bacterium has undergone a significant reduction in size partially as a consequence of genome degradation and reduction caused by continual vertical transmission (Moran 2003). The genome size of *Buchnera* in *A. pisum* (Shigenobu *et al.* 2000) and *Schizaphis graminum* (Hemiptera: Aphididae: Aphidinae: Aphidini) (Tamas *et al.* 2002) is ~640kbp while in the cedar aphid *Cinara cedri* (Hemiptera: Aphididae: Lachninae: Eulachnini) it is even smaller at only 422 kbp (Pérez-Brocal *et al.* 2006). Consequently, the genome size of *Buchnera* is much smaller than of other free-living γ-proteobacteria ($1/7^{th}$ the size of *E. coli* 4639 kbp; Blattner *et al.* 1997). Such reduction in size is characteristic of the genomes of primary symbiont bacteria, which exhibit significant gene loss, large deletions, a rich adenine and thymine content and elevated rates of evolution followed by long periods of stasis (Moran 2003; Moran *et al.* 2008; Moran *et al.* 2009). *Buchnera* exhibits a mutation rate ten times higher than any other bacteria investigated to date (Moran *et al.* 2009) and the same characteristics are observed in *Sulcia* and *Baumannia cicadellinicola*, the co-symbiont bacteria of sharpshooters (McCutcheon and Moran 2007) and *Carsonella ruddii* in psyllids (Tamames *et al.* 2007). As a consequence, *Buchnera* is viable only in its limited niche (Sasaki *et al.* 1991; Douglas 1996). The loss of genes for biosynthesis of cell surface components and non-essential amino acids, regulator genes and genes involved in defence of the cell, that are essential for free living life and the maintenance of genes for replication, transcription and translation and multiple genes for biosynthesis of essential amino acids (Shigenobu *et al.* 2000; Pérez-Brocal *et al.* 2006; Tamames *et al.* 2007) indicates mutual dependence between the aphid and *Buchnera*. The role of *Buchnera* is largely nutritional; it provides the aphid with essential amino acids, which are scarce in the phloem sap upon which it feeds (Douglas 1992; Wilkinson and Douglas 1995; Douglas 1998; Douglas 2003; Sandström and Pettersson 1994; Sandström and Moran 1999; Gündüz and Douglas 2009). Phloem sap provides aphids with quantities of non-essential free amino acids in excess of their demand (Gündüz and Douglas 2009). However, aphid proteins comprise approximately 50% essential amino acids while only

6–20% of phloem sap free amino acids are essential (Sandström and Pettersson 1994). The capacity for aphids to use phloem sap as a food source is attributed to *Buchnera,* which synthesises almost all the essential amino acids in quantities exceeding the daily deficit in the phloem (Gündüz and Douglas 2009). The only exception is methionine for which it is hypothesised that the shortfall is met by aphid usage of the non-protien amino acid 5-methylmethionine in the phloem sap (Gündüz and Douglas 2009). The bacteria are highly cooperative in releasing essential amino acids to the aphid for growth and reproduction and in return are provided with a stable niche and nutrients by the aphid host (Douglas 2003; Birkle *et al.* 2002).

1.3.1.2 The secondary symbionts of aphids.

The second category of symbiont bacteria are facultative and are referred to as secondary symbionts. Unlike the primary endosymbionts they are not restricted to one cell type and are not essential for the survival of the host insect. Secondary symbionts have been found in a variety of cell types including the reproductive organs, the gut and the haemolymph (Griffiths and Beck 1973; McLean and Houk 1973; Fukatsu *et al.* 2000). They are also found in localised concentrations in the secondary mycetocytes, a cell type similar to and in close proximity with the primary mycetocytes that harbour *Buchnera* in the aphid haemocoel (Hinde 1971; Fukatsu *et al.* 2000). Aphid secondary symbionts include *Serratia symbiotica*, *Hamiltonella defensa* and *Regiella insecticola* (Fukatsu *et al.* 2000; Chen *et al.* 2000; Darby *et al.* 2001), which are all γ-proteobacteria and members of the Enterobacteriaceae (Moran *et al.* 2005a). *S. symbiotica* is related to free-living and pathogenic *Serratia* species while *H. defensa* and *R. insecticola* are sister groups with a closer evolutionary relationship to *Photorhabdus* species (Moran *et al.* 2005a). The pea aphid (*Acyrthosiphon pisum*) (Harris) (Hemiptera: Aphididae: Aphidinae: Macrosiphini) is a model for studying facultative symbiosis in insects (Table 1.2), although there are many other examples of insect secondary symbionts including *Arsenophonus* species in psyllids and other arthropods (Dale *et al.* 2006; Hansen *et al.* 2007) (Fig. 1.1). The secondary symbiont bacteria of aphids can affect aphid fitness, and this could influence the dynamics of aphid populations in crop systems (Table 1.2).

Table 1.2: Facultative roles associated with aphid secondary symbiont bacteria.

Aphid species	**Facultative Symbiont**	**Impact on insect fitness**	**Reference**
Acyrthosiphon pisum	*Regiella insecticola*	Increases resistance to the fungal pathogen *Pandora neoaphidis*	Ferrari *et al.* 2001; 2004; Ferrari and Godfray 2003; Scarborough *et al.* 2005
Acyrthosiphon pisum	*Regiella insecticola*	Linked to improved aphid performance on clover (*Trifolium* spp.).	Tsuchida *et al.* 2004 but see Leonardo 2004 and Ferrari *et al.* 2007
Aphis fabae	*Regiella insecticola*	Increases resistance to parasitism by *Aphidius colemani*	Vorburger *et al.* 2010a
Myzus persicae	*Regiella insecticola*	Increases resistance to parasitism by *Aphidius colemani* and *Diaeretiella rapae*	von Burg *et al.* 2008
Acyrthosiphon pisum	*Serratia symbiotica*	Increases thermal tolerance by 'rescuing' *Buchnera*	Montllor *et al.* 2002
Acyrthosiphon pisum	*Serratia symbiotica*	In the absence of *Buchnera* can temporarily fill the empty niche	Koga *et al.* 2003; 2007
Cinara cedri	*Serratia symbiotica*	Compensates for amino acid synthesis capability lacking in *Buchnera*	Pérez-Brocal *et al.* 2006; Gómez-Valero *et al.* 2004; Gosalbes *et al.* 2008
Acyrthosiphon pisum	*Hamiltonella defensa*	Increases resistance to parasitism by *Aphidius ervi*	Ferrari *et al.* 2004; Oliver *et al.* 2003; 2005; 2006; 2008; 2009; reviewed in Oliver *et al.* 2010
Acyrthosiphon pisum	*Hamiltonella defensa*	Increases resistance to parasitism by *Aphidius eadyi*	Ferrari *et al.* 2004
Aphis fabae	*Hamiltonella defensa*	Increases resistance to parasitism by *Lysiphlebus fabarum*	Vorburger *et al.* 2009

1.3.2 Co-diversification of primary symbiotic bacteria with aphids. **(This section is reproduced from the author's published work Clark *et al.* (2010))**

The fossil record suggests that the origin of the primary endosymbiont of insects is ancient and molecular phylogenetic studies have built on information from the fossil record to shed light on the origin, transmission routes and diversification of many primary endosymbiotic bacteria (Munson *et al.* 1991; Baumann *et al.* 1995; Moran *et al.* 2005b). The primary symbiont of aphids, *Buchnera*, is maternally transmitted via the ovaries to the developing embryos (Buchner 1965; Hinde 1971). There is considerable phylogenetic evidence to suggest that transmission of the primary insect symbionts is strictly vertical as co-diversification of primary symbionts and their insect hosts has been demonstrated repeatedly for numerous insect groups including aphids and *Buchnera* (Munson *et al.* 1991; Baumann *et al.* 1995), sharpshooters and *Sulcia* (Moran *et al.* 2005b) and psyllids and *Carsonella* (Thao *et al.* 2000a&b). Even within insect groups there is strong evidence for strict vertical transmission: the aphid genera *Uroleucon* (Clark *et al.* 2000) and *Brachycaudus* (Jousselin *et al.* 2009), for example, show considerable co-diversification of *Buchnera* within close relatives in each aphid genus.

Extreme genome stability is characteristic of the genomes of primary symbionts (Funk *et al.* 2001; Tamas *et al.* 2002). *Buchnera* genomes from unrelated aphid species, for example, show no gene acquisitions or chromosome rearrangements in the past 50–70 million years, indicating the preservation of genome stability of *Buchnera* through several episodes of aphid speciation (Funk *et al.* 2001; Tamas *et al.* 2002). Consequently, given that the evolutionary history of *Buchnera* spans a period that includes many evolutionary shifts in the diet and life cycle of its aphid hosts (Funk *et al.* 2001; Tamas *et al.* 2002), the ecological diversity of aphids is unlikely to be explained by the genetic diversity of *Buchnera*. Indeed the lack of regulatory genes raises the question of whether and how gene expression in *Buchnera* responds to environmental change (Moran and Degnan 2006). Small changes in the genome of the primary symbiont can, however, influence insect fitness and generate genetic variability that is subject to selective pressures. For example, a point mutation in the small heat shock gene (*ibpA*) of *Buchnera* can influence thermal tolerance and reach relatively high frequencies in aphid populations (Dunbar *et al.* 2007). However, the lack of evidence

for horizontal transmission and the highly obligate functional nature of primary symbionts suggest that primary symbionts persist due to their contribution to nutrition rather than their infection capacity or other benefits to host fitness (Wernegreen and Moran 2001). Both vertical and horizontal transmission events are exhibited by the secondary symbionts indicating a more facultative role in insect ecology and evolutionary diversification.

1.3.3 Transmission patterns of secondary symbiotic bacteria in aphids. (This section is reproduced from the author's published work Clark *et al.* (2010))

Secondary symbionts are harboured by a variety of unrelated insect taxa (Darby *et al.* 2001; Russell *et al.* 2003). There is no evolutionary congruence between psyllids and their secondary symbionts (Thao *et al.* 2000a), or between several types of the arthropod secondary symbiont *Rickettsia* and their insect hosts (Weinert *et al.* 2009), although there is some evidence that *S. symbiotica* co-diversified with aphids in the subfamily Lachninae (Lamelas *et al.* 2008; Burke *et al.* 2009). Secondary symbionts distribution is erratic within and between insect taxa and they are not consistently associated with any one species (Tsuchida *et al.* 2002; Haynes *et al.* 2003; Hansen *et al.* 2007). For example, the aphid secondary symbiont *H. defensa* is commonly but not ubiquitously found in pea aphid and is closely allied to a symbiont of the whitefly *Bemisia tabaci* (Darby *et al.* 2001). The labile distribution of secondary symbionts across the insect taxa is thought to reflect repeated horizontal transmission events between and within different insect groups (Sandström *et al.* 2001).

According to phylogenetic analysis, secondary symbionts have been acquired independently by a wide variety of herbivorous insects, supporting the view that they can be transmitted horizontally as well as vertically (Thao *et al.* 2000a; Sandström *et al.* 2001; Russell *et al.* 2003). In addition, the lack of genetic divergence between secondary symbionts harboured by different groups of insects suggests that horizontal transmission across taxa occurred far more recently in evolutionary time than the origin of ancient primary symbioses (Fukatsu *et al.* 2000; Russell *et al.* 2003). To date, however, all attempts to demonstrate horizontal transmission of secondary symbionts via an ecological route using laboratory experiments have been unsuccessful. Attempts to establish horizontal transmission between sympatric infected and uninfected aphid

lines via a common host plant or aphid parasitoids have been unsuccessful (Chen *et al.* 2000). However, transfer of secondary symbionts to insect hosts via artificial diets and microinjection, resulting in stable vertical transmission, has been achieved in the laboratory (Russell and Moran 2005; Pontes and Dale 2006). Current opinion suggests that occasional horizontal transmission events between insects or via the environment are essential for the establishment of facultative secondary infection in wild populations of aphids, which are then maintained by high levels of vertical transmission and account for the multiple evolutionary origins of secondary symbionts across insect taxa (Sandström *et al.* 2001; Russell *et al.* 2003). The fitness benefits of harbouring secondary 'facultative' symbionts in aphids are well documented but fitness costs are also associated with secondary symbiont infection. Infection with *S. symbiotica* in the blue alfalfa aphid *Acyrthosiphon kondoi* (Shinji) (Hemiptera: Aphididae: Aphidinae: Macrosiphini), for example, results in prolonged developmental times and reduced fecundity and longevity (Chen *et al.* 2000). Harbouring secondary symbionts could therefore result in fitness trade-offs determined by selective pressures imposed by the environment. In natural populations selection pressures imposed by predators and parasitoids, fluctuations in temperature or nutrient provisioning by plants will vary altering the costs or benefits associated with harbouring secondary symbionts (Russell and Moran 2006). In addition, aphids can also become infected with other free-living bacterial species that are present within the environment and become established in the aphid gut, including *Erwinia* (Harada *et al.* 1997), *Pseudomonas* (Harada *et al.* 1996) and *Spiroplasma* (Fukatsu *et al.* 2001) species. Infection with these bacteria can have pathogenic effects, reducing aphid size and fecundity (Harada and Ishikawa 1997; Grenier *et al.* 2006). The mode of transmission of these bacteria is likely to be predomiantly horizontal rarther than vertical and as a consequence their vulnerability to selective pressures will differ from the commonly studied pea aphid secondary symbionts. Thus, understanding the fitness effects conferred by bacteria and their evolutionary dynamics are key to understanding the mechanisms that maintain bacterial infections in aphid populations.

1.3.4 Serratia symbiotica, thermal tolerance and the primary symbiosis.

Heat stress can disrupt the aphid–*Buchnera* symbiosis and nutrient provisioning, thus suppressing reproduction (Douglas 1998). Prolonged exposure to temperatures greater than 30°C can inhibit reproduction by adults while nymphs exposed to heat stress in early development fail to reproduce in adulthood (Satar *et al.* 2005). In California, the pea aphid harbours *S. symbiotica* in varying frequencies throughout the state (Chen and Purcell 1997). A high incidence of *S. symbiotica* has been reported in the summer months suggesting its prevalence may be related to elevated temperatures (Montllor *et al.* 2002). The fecundity of pea aphid lines infected with *S. symbiotica* was considerably greater under heat stress (exposure to 39ºC) compared with uninfected control pea aphid lines (Montllor *et al.* 2002). Additionally, the number of mycetocytes (containing *Buchnera*) normally associated with heat stress was significantly reduced in pea aphid lines containing *S. symbiotica* indicating that *S. symbiotica* was able to prevent disruption of the primary symbiosis under high temperatures (Montllor *et al.* 2002). The positive effects of *S. symbiotica* might be caused by the delivery of protective metabolites to *Buchnera* after heat exposure (Burke *et al.* 2010). Thus, *S. symbiotica* could be highly beneficial to aphids exposed to high temperatures by effectively 'rescuing' *Buchnera* (Montllor *et al.* 2002; Chen *et al.* 2000). There was, however, no correlation between the mean temperature of each geographical location in California and the frequency of *S. symbiotica* infection, although *S. symbiotica* might protect the aphid from heat stress during periods of summer heat (Montllor *et al.* 2002).

In artificially manipulated situations *S. symbiotica* can partially compensate for the loss of *Buchnera* by physiologically and cytologically taking over its niche. When *Buchnera* is eliminated via antibiotic treatment *S. symbiotica* can infect the cytoplasm of the mycetocytes which would normally house *Buchnera* (Koga *et al.* 2003; Koga *et al.* 2007). Replacement of *Buchnera* by *S. symbiotica* indicates a mechanism for evolution of novel obligate endosymbioses with previously facultative bacteria (Koga *et al.* 2003). In the cedar aphid the size of the *Buchnera* genome has been significantly reduced compared to other aphid species and has lost the genes for synthesis of some essential micronutrients, including tryptophan (Pérez-Brocal *et al.* 2006). However, *C. cedri* also harbours large numbers of the secondary symbiont *S. symbiotica*, which can synthesise the missing micronutrients (Gómez-Valero *et al.* 2004; Gosalbes *et al.* 2008)

presumably compensating for the lack of *Buchnera* function. A long-term evolutionary relationship has been suggested between *S. symbiotica* and aphids in the genus *Cinara* and subfamily Lachninae as a whole, as the clade of *S. symbiotica* associated with the Lachninae appears to have established independently of the *S. symbiotica* associated with other aphid subfamilies in the family Aphididae (Lamelas *et al.* 2008). *S. symbiotica* in the clade associated with the Lachninae subfamily showed faster evolution of the protein coding *atpD* gene and were similar in size and shape to *Buchnera* (Lamelas *et al.* 2008), suggesting that functional replacement of *Buchnera* by *S. symbiotica* might be occurring in Lachninae aphids. However, some *Cinara* species lacked any symbiont other than *Buchnera*, suggesting that the requirement for *S. symbiotica* is not universal (Burke *et al.* 2009). The relationship between the facultative *S. symbiotica* and the cedar aphid illustrates the extent to which relationships with secondary bacteria can influence a number of aspects of aphid ecology and even provide functions that are essential for aphid survival.

1.3.5 Secondary symbionts and the aphid–plant interaction.

Several studies have suggested that the secondary facultative bacteria of aphids could influence aphid performance in relation to aphid nutrition and plant use and determine the range of host plants upon which aphids feed and reproduce (Table 1.3) (Chen *et al.* 2000; Wilkinson *et al.* 2001; Darby *et al.* 2003; Ferrari *et al.* 2004; Tsuchida *et al.* 2004; Tsuchida *et al.* 2006; reviewed in Clark *et al.* 2010). Although a physiological study of pea aphid on chemically-defined diets of different sucrose and amino acid contents did not identify any link between infection with *H. defensa*, *S. symbiotica* or *R. insecticola* and aphid performance (Douglas *et al.* 2006a), *S. symbiotica* can synthesise essential amino acids (Koga *et al.* 2003; Koga *et al.* 2007; Pérez-Brocal *et al.* 2006; Gómez-Valero *et al.* 2004; Gosalbes *et al.* 2008). By contrast *H. defensa* is not able to synthesise eight of the essential amino acids and is therefore dependent on *Buchnera* for these nutrients (Degnan *et al.* 2009). Facultative symbionts with the capacity to supply the aphid with essential nutrients might therefore enable aphids to feed on nutritionally-poor plants while those that lack a nutritional capacity might compete with the host insect or indeed *Buchnera* for resources (Clark *et al.* 2010). Such conflicts in symbiotic associations are common but they are generally

managed and contained (Douglas 2008). Trade-offs in bacterial infection versus nutrition could however drive aphids onto different nutritionally rich host plants thus influencing host plant specialisation (Janson *et al.* 2008; Clark *et al.* 2010). Moreover increasing evidence suggests that the effect of facultative endosymbionts on plant utilisation by aphids is a complex interaction between aphid and endosymbiont genotype and acquired resistance to natural enemies (Clark *et al.* 2010).

There is considerable evidence for host plant specialisation in pea aphid that is attributed to aphid genotypic effects (Hawthorne and Via 2001; Ferrari *et al.* 2006; Ferrari *et al.* 2008). However, infection with facultative secondary symbiont bacteria might also influence pea aphid specialisation on different host plants (Simon *et al.* 2003; Ferrari *et al.* 2004; Ferrari *et al.* 2007; reviewed in Clark *et al.* 2010). Infection with the secondary facultative symbiont *R. insecticola* is often linked to host plant specialisation (Simon *et al.* 2003; Tsuchida *et al.* 2004; Ferrari *et al.* 2004; Frantz *et al.* 2009). In Japan the pea aphid feeds and reproduces on vetch (*Vicia sativa*) and white clover (*Trifolium pratense*) and infection with *R. insecticola* is more common in the northern regions where vetch is rare and white clover is abundant, while in areas where vetch and white clover are sympatrically available *R. insecticola* frequency is higher in aphids on white clover (Tsuchida *et al.* 2004). Elimination of *R. insecticola* by antibiotic injection caused a 50% reduction in pea aphid fecundity on white clover while fecundity on vetch remained unchanged. Reintroduction of *R. insecticola* by haemolymph injection allowed recovery of fecundity on white clover, indicating the potential for *R. insecticola* to favour specialisation on white clover through nutritional fitness benefits (Tsuchida *et al.* 2004). However, subsequent studies have revealed either aphid genotypic diversity (Leonardo 2004) or an interaction between aphid and bacterial genotypes (Ferrari *et al.* 2007) determined specialisation on clover rather than infection with *R. insecticola* alone.

The maintenance of high frequencies of *R. insecticola* in pea aphid populations specialised on clover must therefore not simply be related be to aphid nutrition, but linked to as yet unidentified aphid traits or fitness trade-offs (Clark *et al.* 2010). *R. insecticola* has been associated with increased aphid resistance to the fungal pathogen *Pandora neoaphidis* in pea aphid (Scarborough *et al.* 2005) and to the parasitoid wasp *Aphidius colemani* (Viereck) (Hymenoptera: Braconidae: Aphidiinae) in *Myzus*

persicae (Sulzer) (Hemiptera: Aphididae: Aphidinae: Macrosiphini) (von Burg *et al.* 2008) and *Aphis fabae* (Scopoli) (Hemiptera: Aphididae: Aphidinae: Aphidini) (Vorburger *et al.* 2010a). Conversely, under certain circumstances, such as exposure to high temperatures, infection with *R. insecticola* has been demonstrated to be a liability for the aphid host (Russell and Moran 2006). Therefore the presence of *R. insecticola* could be a consequence of increased natural enemy pressure on clover, which increases the need for the aphid to maintain a symbiont that might otherwise be costly (Clark *et al.* 2010). Thus, evidence for secondary symbiont mediated host plant specialisation (Table 1.3) might directly reflect differences between host plants in their association with aphid natural enemies or other fitness trade-offs imposed by the environment (Clark *et al.* 2010).

Table 1.3: Plant affiliation and association with facultative symbiont bacteria of aphids. **(Table produced by A.J. Karley for the author's published work Clark *et al.* (2010))**

Insect species	Facultative symbiont	Plant species	Observation	References
Acyrthosiphon pisum	*Regiella insecticola*	*Trifolium pratense* (Red clover)	High frequency on red clover in France, UK	Simon *et al.* 2003 Ferrari *et al.* 2004 Frantz *et al.* 2009
	Regiella insecticola	*Trifolium repens* (White clover)	High frequency on white clover in California, Japan	Leonardo and Muiru 2003 Tsuchida *et al.* 2004
	Regiella insecticola	*Trifolium* spp.	Improved performance on clover compared to alfalfa and vetch	Leonardo and Muiru 2003 Tsuchida *et al.* 2004 (but see Leonardo 2004 and Ferrari *et al.* 2007)
	Hamiltonella defensa	*Medicago sativa* (Alfalfa) *Lotus pedunculatus* (Bird's foot trefoil)	Higher frequency of symbiont on alfalfa and Lotus	Simon *et al.* 2003 Ferrari *et al.* 2004 Frantz *et al.* 2009 (but see Darby *et al.* 2003)
	Serratia symbiotica *Rickettsia*	*Pisum sativum* (Pea) *Vicia faba* (Faba bean)	High frequency on pea and bean	Simon *et al.* 2003 Ferrari *et al.* 2004 Frantz *et al.* 2009
	Rickettsia	*Vicia faba* (Faba bean)	Reduced fecundity on bean and clover	Simon *et al.* 2007 Chen *et al.* 2000
Acyrthosiphon pisum *Acyrthosiphon kondoi*	*Rickettsia* *Serratia symbiotica*	*Pisum sativum* (Pea) *Vicia faba* (Faba bean) *Medicago sativa* (Alfalfa) *Lathyrus odorata* (Sweet pea)	Variable effects on aphid fitness depending on aphid genotype and plant species	Chen *et al.* 2000

1.3.6.1 Secondary symbionts and resistance to parasitism.

Aphids depend on *Buchnera* symbionts for the provision of essential nutrients for survival, indicating that the fitness of developing parasitoid larva and aphid symbionts are likely to be highly interlinked. Cloutier and Douglas (2003) showed that parasitised aphids containing a growing parasitoid larva had more *Buchnera*-containing mycetocytes with a greater overall biomass. Aphid embryos were of lower mass in parasitised hosts, when compared to unparasitised hosts, indicating partitioning of resources towards the developing parasitoid (Cloutier and Douglas 2003). Research investigating the fitness of emergent parasitoids from insects harbouring secondary bacteria has focused mainly on the interaction between the reproductive symbiont *Wolbachia* and the fruit fly *Drosophila* (Diptera: Drosophilidae) in which some fitness traits of *Drosophila* parasitoids are reduced by *Wolbachia* infection (Dobson *et al.* 2002; Kraaijeveld *et al.* 2002; Mouton *et al.* 2004; Fytrou *et al.* 2006). In the *Drosophila* parasitoid–*Wolbachia* system there is evidence for co-evolution between host resistance and parasitoid virulence (Kraaijeveld *et al.* 1998). Miao *et al.* (2004) examined the effect of eliminating *Buchnera*, using the antibiotic rifampicin, on parasitoid development and reproduction, and found that in the absence of *Buchnera* the larval size, growth rate, rate of emergence and number of progeny were significantly reduced. However, the main focus of research on aphid–bacteria–parasitoid interactions has not been the effects of bacterial infection on the fitness of parasitoids, but instead has been the increased resistance to parasitism conferred by the facultative symbionts of some aphid species.

The three secondary facultative endosymbionts of aphids *H. defensa*, *S. symbiotica* and *R. insecticola*, have very different effects on the ability of the aphid to resist parasitism (reviewed in Oliver and Moran 2009 and Oliver *et al.* 2010). Until recently the evidence suggested that *H. defensa*, and to a lesser extent *S. symbiotica*, were predominantly responsible for enhancing resistance to parasitoids, but it has now been established that *H. defensa* carries a bacteriophage (APSE) (van der Wilk *et al.* 1999) which encodes toxins responsible for the defensive function (Oliver *et al.* 2009). The APSE phage which carries a gene encoding cytolethal distending toxin (*cdtB*) that has been recorded from several mammalian pathogens, and which acts by breaking the eukaryotic cell cycle (Moran *et al.* 2005c) is now thought to be the behind phenotypic resistance to parasitism in aphids

(Degnan and Moran 2008b). In *A. pisum* parasitised by *A. ervi* the presence of the toxin-encoding bacteriophage (APSE-3) conferred a 10-fold greater resistance to parasitism compared to infection with *H. defensa* alone (Oliver *et al.* 2009). These results suggested that any trait encoded by a bacteriophage that benefits the host can lead to rapid spread of phage-associated bacterial symbionts in host populations (Clark *et al.* 2010). Recently however, it has also been demonstrated that a single strain of *R. insecticola*, not carrying the APSE bacteriophage, confers very high levels of resistance to parasitoids in a single Australian clone of the peach–potato aphid *M. persicae* (von Burg *et al.* 2008) and *A. fabae* (Vorburger *et al.* 2010a). Consequently, there might be additional complexity in the maintenance and evolution of bacteria-mediated resistance to parasitism in aphids.

1.3.6.2 Bacteria-mediated fitness trade-offs and genetic effects in resistance to parasitism.

Research into symbiont-mediated resistance to parasitism in aphids indicates that a set of generalist ecological strategies can be conferred by symbiont bacteria to facilitate their maintenance in aphid populations, with a lack of host specificity which should lead to greater frequencies, if not fixation, in natural populations (Clark *et al.* 2010). The fact that they have not reached fixation suggests that infection with bacterial symbionts might be constrained by fitness trade-offs associated with bacterial infection (Russell and Moran 2006; Gwynn *et al.* 2005; Clark *et al.* 2010). For example, the early lifetime fecundity in pea aphid parasitised by *A. ervi* has been shown to be reduced in more resistant aphid genotypes (Gwynn *et al.* 2005). Additionally, double infection with the symbionts *H. defensa* and *S. symbiotica* has been shown to confer greater resistance to parasitism than either single infection (Oliver *et al.* 2006). However, double infections are rare in natural populations of *A. pisum* suggesting that there are severe fitness costs, probably reductions in fecundity, which prevent the establishment of multiple infections (Oliver *et al.* 2006). The patchy distribution of natural aphid populations results in some populations naturally avoiding parasitism (Hufbauer 2002; Fathipour *et al.* 2006; Pareja *et al.* 2008), which could also reduce selection in favour of costly infections (Clark *et al.* 2010).

Aphid genetic effects as opposed to symbiont mediated defences might also explain variation in resistance to parasitism. The different host races of pea aphid found in North

America vary in their susceptibility to parasitism (Henter 1995; Henter and Via 1995). These differences were originally attributed to genotypic effects but could equally be mediated by association with symbiotic bacteria. To eliminate the effect of genotype and determine whether secondary symbiont complement alone determined variation in aphid resistance to parasitism, haemolymph containing *H. defensa*, *S. symbiotica* or *R. insecticola* was transferred from infected to uninfected pea aphid lines (Oliver *et al.* 2003). In a controlled aphid genetic background, secondary symbiont infection with *H. defensa* promoted resistance to the parasitoid by causing elevated mortality amongst the developing *A. ervi* larvae, indicating that genetic differences in aphid lines were not responsible for the observed increase in resistance to parasitism (Oliver *et al.* 2003). The parasitoids in the experiment exhibited no discrimination towards hosts containing the resistance-conferring symbionts, which is surprising given the high cost to parasitoid fitness (Oliver *et al.* 2003). Parasitoids were however, maintained throughout on a symbiont-free aphid line and therefore had no experience of *H. defensa*-infected aphids (Oliver *et al.* 2003). It is likely therefore that a more prolonged association must exist between an aphid clone, one or more secondary symbionts, and a parasitoid before a co-evolutionary relationship that involves such discrimination can become established (Clark *et al.* 2010).

1.3.7 The role of the secondary symbionts of aphids within crop systems.

The combined effects of environmental factors like temperature and host plant quality are likely to have variable outcomes for the relationship between the aphid, parasitoid and symbiont. For example, resistant pea aphid clones that contain *H. defensa* are highly susceptible to parasitism at temperatures above 25ºC, indicating that temperature is not only important for parasitoid development but for the aphid–endosymbiont–parasitoid interaction as well (Bensadia *et al.* 2006). *Wolbachia*, the primary symbiont of parasitoids, is killed by exposure to high temperatures, suggesting that temperature-induced mortality of endosymbionts could explain patterns of resistance to parasitism in aphids (Werren 1997). More recently it was shown that not only did increasing temperature reduce the resistance conferred by *H. defensa* but that super-infected clones, which harboured a newly identified facultative symbiont of pea aphid designated PAXS (pea aphid X-type symbiont) in addition to *H. defensa*, retained high levels of resistance to parasitism under heat stress

(Guay *et al.* 2009). Even when not heat stressed, double infected clones showed much higher resistance levels than those conferred by *H. defensa* alone, suggesting a strong synergy between the two symbionts (Guay *et al.* 2009).

Host plant quality could also influence the aphid–parasitoid symbiont interaction. The growth rate of *A. fabae* is slowed when it feeds on *Lamium purpureum*, a plant low in amino acid content of phloem sap compared with *Vicia faba*, a plant high in amino acid content (Chandler *et al.* 2008). *A. fabae* development is also slower on synthetic diets with low amino acid content than on *V. faba* (Chandler *et al.* 2008). Elevated densities of the secondary symbionts *S. symbiotica*, *R. insecticola* and *H. defensa* are associated with aphids feeding on *L. purpureum* and synthetic diets with low amino acid content. The low nutrient concentration in *L. purpureum* appeared to promote deleterious traits in the secondary symbionts and disturb insect control over bacterial populations. It is possible that the impact of diet quality on symbiotic bacteria and their aphid host could have 'knock-on' effects on parasitoid fitness. In addition, availability of pollen, nectar and aphid honeydew resources from different plant types, towards which parasitoids exhibit strong preferences, varies significantly between host plants (Wackers 2004; Wackers *et al.* 2008). Consequently, host plant selection by the aphid could influence resistance to parasitism and symbiont-mediated protection against natural enemies (Clark *et al.* 2010).

1.4 Aims of this Study.

1.4.1 Limitations to current knowledge.

It is likely that aphid fitness and the consequences for arable food web interactions will depend critically on the bacterial assemblages that they harbour, the spatial structure of the habitat and the abiotic and biotic conditions of their environment. To date most studies have focused on the pea aphid, which it is not an economically important pest in Scotland. New studies of alternative aphid species will provide important information about aphid–parasitoid–bacteria interactions that should be more relevant to Scottish agriculture. Other aphid species might harbour different bacterial types, which could differ in their mode of transmission and consequently their evolutionary dynamics and could have an as yet undetermined effect on aphid populations. The secondary symbionts, *H. defensa*, *S.*

symbiotica or *R. insecticola* have not been detected previously in cabbage aphid, and these aphids might harbour a very different bacterial complement compared to pea aphids. In addition, the population dynamics of parasitoid wasps are poorly understood. Increasing our understanding of the constraints on parasitoid population processes, including environmental factors and interactions with bacteria symbionts, would shed light on many aspects of the aphid–parasitoid interaction and indicate ways in which their application as biocontrol agents could be optimised. Previous research indicated high levels of host aphid specificity among parasitoid populations (e.g. Antolin *et al.* 2006) thus it is likely that parasitoid wasps have become specialised to particular aphid–plant associations. However, taxonomic records for parasitoid wasps are limited and few studies have examined the genetic relatedness of parasitoid sub-populations that attack different aphid–plant associations. In addition, little is known about the effect of the bacterial complement of the aphid on parasitoid fitness. Infection with pathogenic bacteria associated with the aphid gut, for example, has been shown to reduce aphid performance (Grenier *et al.* 2006; Harada and Ishikawa 1997) which could affect the fitness of parasitoid populations and influence specialisation of parasitoids on different aphid hosts. The parasitoid could, for example, exploit the immune system of an aphid that has been compromised by infection with pathogenic bacteria. Conversely, infection with pathogenic bacteria could be detrimental to parasitoid embryo development which is dependent on the partitioning of resources from host tissues. Parasitoid wasps appear to be one of the least studied insect groups despite their importance as natural enemies. Many mathematical models built to predict aphid population dynamics in crop systems did not include the fitness effects of aphid bacterial complement and the aphid–parasitoid–symbiont interaction (Parry *et al.* 2006; Stark and Acheampong 2007), consequently there is potential to build more robust models that include these additional fitness parameters. Such models would be a useful tool for predicting aphid population dynamics with the aim of reducing crop damage caused by aphids in arable systems.

1.4.2 Aim of this study.

The aim of this study was to address some of the gaps in our understanding of aphid population dynamics in crop systems in Scotland by focusing on the interaction between

the arable pest the cabbage aphid, its bacterial complement and its parasitoid wasp *D. rapae*. Using molecular methods to characterise and quantify the bacteria associated with the cabbage aphid formed a basis for determining the effects of different bacterial types on both the fitness of the cabbage aphid and *D. rapae* both in the glasshouse and in a field situation. The four key objectives of the study were as follows:

1) Characterise the bacteria associated with cabbage aphid lines collected in Scotland.
2) Develop a molecular technique to screen multiple cabbage aphid lines to provide a basis for fitness experiments.
3) Compare the fitness of several cabbage aphid lines with varying bacterial complements.
4) Determine whether there is any effect of cabbage aphid bacterial complement on the fitness of *D. rapae* parasitoids.

2. Molecular characterisation of the bacterial complement of the cabbage aphid.

2.1 Introduction

2.1.1 Insects form numerous associations with bacteria.

Insects have formed relationships with a wide diversity of bacteria, which can be symbiotic, pathogenic or have no detectable effect on the fitness of the insect (Buchner 1965). Associations between insects and bacteria can take a number of different forms often involving multiple bacterial players and varied life styles. Bacteria that are found extracellularly in the gut of termites, for example, are symbiotic and play a role in the degradation of cellulose (Tokuda and Watanabe 2007). Other bacteria have formed an obligate intracellular symbiosis with their insect host and fulfil an essential role upon which the host depends for survival, such as the provision of certain essential amino acids that the host is unable to synthesise (Douglas 1998; 2009). Bacteria can also form facultative symbioses with insects in which they are not essential for the survival of the insect but can confer fitness benefits that help protect the insect against natural enemies (Oliver *et al.* 2003; Ferrari *et al.* 2004). The relationship between aphids and their symbiotic bacteria has been particularly well studied. Almost all aphid species harbour the bacterial 'primary' endosymbiont, *Buchnera aphidicola*, (Buchner 1965; Munson *et al.* 1991) upon which aphids depend for the synthesis of essential amino acids (Douglas 1998; 2009; Gündüz and Douglas 2009; Wilson *et al.* 2010). *Buchnera* are integral to the physiology of the host insect and are located in the cytoplasm of hypertrophied cells specialised for endosymbiosis called 'mycetocytes' (also referred to as bacteriocytes) which reside in a specialised organ called a 'mycetome' (also referred to as a 'bacteriome') (McLean and Houk 1973; Buchner 1965). *Buchnera* is a member of the γ-3 subdivision of the Proteobacteria and forms a phylogenetically distinct clade within the Enterobacteriaceae (Munson *et al.* 1991). In addition to *Buchnera* aphids also harbour multiple other types of secondary or facultative bacterial types including intracellular symbionts and extracellular free-living bacteria (Harada and Ishikawa 1993; Grenier *et al.* 1994; Harada *et al.* 1996).

2.1.2 Several types of aphid secondary bacteria are known to influence aphid fitness.

Three types of secondary symbiont bacteria have been particularly well characterised in the pea aphid *(A. pisum*), namely *S. symbiotica*, *H. defensa* and *R. insecticola* (Chen *et al.* 2000; Fukatsu *et al.* 2000; Sandström *et al.* 2001; Darby *et al.* 2001). They are found in localised concentrations in the 'secondary mycetocytes', a cell type similar to and in close proximity with the 'primary mycetocytes' that harbour *Buchnera* in the aphid haemocoel (Hinde 1971; Douglas 1998; Fukatsu *et al.* 2000; Sandström *et al.* 2001). Other types of secondary bacteria that have been characterised in aphids include *Arsenophonus* species (Russell *et al.* 2003), *Rickettsia* species (Chen *et al.* 2000; Sakurai *et al.* 2005), *Spiroplasma* species (Fukatsu *et al.* 2001) and *Erwinia* species (Grenier *et al.* 1994; Harada *et al.* 1996; Harada and Ishikawa 1997). These bacteria are extracellular and are associated with the aphid gut and other tissues (Harada and Ishikawa 1993; Grenier *et al.* 1994; Harada *et al.* 1996; Harada *et al.* 1997). Infection with *Erwinia* and *Spiroplasma* species can reduce pea aphid fitness (Harada and Ishikawa 1997; Fukatsu *et al.* 2001; Grenier *et al.* 2006).

In contrast, the secondary symbionts *S. symbiotica*, *H. defensa* and *R. insecticola* are thought to confer positive fitness effects on their aphid hosts. *H. defensa* has been linked to increased resistance to parasitism (Oliver *et al.* 2003; 2005; 2006; 2009; 2010), *R. insecticola* appears to influence host plant specificity (Tsuchida *et al.* 2004) and protect against fungal pathogens (Scarborough *et al.* 2005), and *S. symbiotica* can increase thermal tolerance (Montllor *et al.* 2002) and may even partially compensate for the loss of the primary symbiont *Buchnera* (Koga *et al.* 2003; Pérez-Brocal *et al.* 2006; Lamelas *et al.* 2008; Gosalbes *et al.* 2008). However, unlike *Buchnera*, aphid secondary bacteria are not harboured universally within and between aphid species. Instead, their distribution is far more sporadic suggesting that the evolutionary origin of their association with aphids is relatively recent (Sandström *et al*. 2001; Tsuchida *et al.* 2002; Haynes *et al.* 2003; Russell *et al.* 2003; Moran *et al.* 2008). Close relatives of the secondary symbionts are found in other insect taxa: *H. defensa* is closely allied to a secondary symbiont found in whitefly (*Bemisia tabaci*), for example (Darby *et al.* 2003; Chiel *et al.* 2009). Secondary bacteria could be transmitted horizontally between or within taxa; any resultant fitness benefit to the host will select for vertical transmission to progeny (Darby and Douglas 2003; Sandström

et al. 2001; Russell *et al.* 2003). These postulated horizontal transmission events could have a profound influence on aphid phenotype and determine the survival of aphid lines under fluctuating selective pressures imposed by the environment (Oliver *et al.* 2008). Consequently, the secondary symbiont bacteria of aphids are likely to be key players influencing the fitness of aphids and their competence as arable pests and characterising these bacteria forms the basis for studying their effects on aphid fitness.

2.1.3 Molecular methods can be used to characterise bacteria in insects.

Historically bacteria were characterised based on their morphological, biochemical and physiological properties but with the development of molecular methods such as polymerase chain reaction (PCR) and cloning and sequencing there is now a wealth of sequence data available to use for phylogenetic characterisation based on DNA sequence (Naum *et al.* 2008). To use phylogenetic analysis to identify bacterial groups, a region of DNA that exhibits sufficient sequence variation to differentiate between bacterial types is required. Small housekeeping genes that are shared by all bacteria have been particularly useful in determining phylogenetic relationships in bacteria (Brown *et al.* 2000). The 16S ribosomal subunit is one such housekeeping gene that is commonly used for phylogenetic characterisation of the symbiont bacteria of insects (Fukatsu and Nikoh 1998; Fukatsu 2001; Fukatsu *et al* 2000; 2001; Darby *et al.* 2001; Dunn and Stabb 2005; Sakurai *et al.* 2005). It allows classification from the phylum to genus and species level (Wertz *et al.* 2003). 16S rRNA sequence analysis formed the basis of the first molecular identification of two distinct prokaryotic intracellular symbionts in the pea aphid (*A. pisum*) (Unterman *et al.* 1989). For comprehensive characterisation of symbiont bacteria, phylogenetic analysis of 16S sequence is combined with microscopy techniques such as fluorescent *in situ* hybridisation (FISH) to localise the bacteria to specific tissues within the insect (Fukatsu 2001; Fukatsu *et al* 2000; 2001; Darby *et al.* 2001; Sakurai *et al.* 2005).

The secondary bacteria have been fully characterised using 16S sequence in only a few aphid species including *A. pisum* (Unterman *et al.* 1989; Fukatsu *et al.* 2000, 2001; Darby *et al.* 2001; Sandström *et al.* 2001), *Aphis glycines* (Wille and Hartman 2009), *Uroleucon* species (Sandström *et al.* 2001), *Yamatocallis* species (Fukatsu 2001), and *Cinara* species (Lamelas *et al.* 2008; Burke *et al.* 2009). Other studies have used diagnostic

methods, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and diagnostic PCR assays, to test for the presence of *H. defensa*, *S. symbiotica* and *R. insecticola* across a number of aphid species within the family Aphididae without investigating the full spectrum of bacteria which may be associated with each aphid species (Tsuchida *et al.* 2002; Haynes *et al.* 2003; Russell *et al.* 2003; Chandler *et al.* 2008). Consequently, the degree of diversity of bacteria associated with many aphid species and the extent to which they influence aphid fitness is not clear. At the outset of the study little was known about the bacterial complement of the cabbage aphid (*Brevicoryne brassicae*), a common arable pest in Scotland. In a single cabbage aphid line from the United States, *H. defensa*, *S. symbiotica* and *R. insecticola* were not detected (Russell *et al.* 2003). Cabbage aphid differs from other aphid species by feeding obligately on crucifers that are high in glucosinolates (Bones and Rossiter 1996). Cabbage aphids have evolved independantly their own myrosinase capable of hydrolysing a number of glucosinolates including sinigrin as a defence against natural enemies (Jones *et al.* 2002; Bridges *et al.* 2002). The ability to exploit and mimic the crucifer plant defences is unique to cabbage aphid among the aphid species in which bacterial complement has previously been characterised. As aphid physiology could influence bacterial infection, we have no *a priori* assumptions about the presence and composition of the bacterial complement of this aphid species. The aim of this study was to use 16S sequencing to characterise the bacteria associated with the cabbage aphid (*B. brassicae*) as a basis for testing the influence of any detected secondary bacteria on both the fitness of the aphid and of its parasitoid *D. rapae*.

2.2 Materials and Methods

2.2.1 Experimental aphids

Populations of cabbage aphid *B. brassicae* were collected from the field in October 2006 and July and August 2007 from brassica plants in Tayport, Newport and Letham in Fife, Dundee and Invergowrie in Tayside and Snainton in North Yorkshire (Table 2.1). Field collected aphid populations were established as clonal lines with single nymphs from each original population. Since aphids reproduce by parthenogenesis all individuals descended from the original nymph were genetically identical. The original mixed–clone populations were also maintained in culture. In addition several pea aphid lines with known

secondary infections were also maintained as clonal cultures (Table 2.2) to use as positive controls for diagnostic tests for *H. defensa, S. symbiotica* and *R. insecticola* (Section 2.2.4.1) and in Chapter 5 for analysis of *Buchnera* density. The pea aphid lines were provided by Professor Angela Douglas (University of York) and Dr Glen Powell (Imperial College). An overview of the different cabbage aphid lines used for each experiment within each chapter is presented in Table 2.3.

All aphids were reared in controlled temperature rooms at 19ºC with 16 hours light: 8 hours dark on excised leaves. Aphid infested leaves were housed in stacked pairs of clear plastic culture cups (No. 16 Clear Plastic Cups and Lids, A&W Gregory Co. Ltd) (Plate 2.1) The outer cups held approximately 2 cm of water and the inner cups had 1.5 cm diameter holes in the base through which leaf stalks were inserted. A plastic lid was used to secure a square of fine mesh gauze to seal the pot. Aphids were sub-cultured twice weekly, at which time the culture cups were cleaned, the water was changed and old heavily infested plant material was discarded and exchanged for fresh plant material. Cabbage aphid lines were maintained on brussels sprout leaves *Brassica oleracea* cultivar 'Evesham Special' (B&Q Product No. 311132 Brussels Sprout cv. Evesham Special) while broad bean *Vicia faba* cultivar 'The Sutton' was used to maintain the pea aphid lines. All plant material was grown in a glasshouse from seed on insecticide free compost (sand–perlite–peat mix containing N:P:K 17:10:15, William Sinclair Horticulture, Lincoln, U.K).

Table 2.1: Code, collection date, location and collection host plant for experimental cabbage aphid lines.
***ELC0606P and ELC0604P are not the same aphids as ELC0604 and ELC0606. (Grid references Landranger Ordnance Survey Maps 54, 59 and 100, 1:50,000)**

Code	Date Collected	Location	Grid Reference	Brassica Variety
ELC0603	10/10/2006	Balmullo, Fife	NO418185	Kale
ELC0604	11/10/2006	Tayport, Fife	NO457285	Cabbage
ELC0605	12/10/2006	Dundee. Tayside	NO393312	Brussels Sprout
ELC0606	12/10/2006	Letham, Fife	NO297146	Broccoli
ELC0607	12/10/2006	Tayport, Fife	NO457285	Purple Sprouting Broccoli
ELC0608	13/10/2006	Wormit, Fife	NO397255	Broccoli
ELC0610	13/10/2006	Balmullo, Fife	NO418185	Kale
ELC0611	17/10/2006	Dundee, Tayside	NO393312	Cabbage
ELC0612	17/10/2006	Dundee, Tayside	NO393312	Brussels Sprout
ELC0613	20/10/2006	Tayport, Fife	NO457285	Brussels Sprout
ELC0615	20/10/2006	Balmullo, Fife	NO418185	Brussels Sprout
ELC0618	24/10/2006	Dundee, Tayside	NO386314	Cauliflower
ELC0619	25/10/2006	Dundee, Tayside	NO386314	Turnip
ELC0701	10/07/2007	Invergowrie, Tayside	NO342298	Oilseed Rape
ELC0703	20/08/2007	Tayport, Fife	NO457285	Brussels Sprout
ELC0801	21/07/2008	Snainton, Yorkshire	SE824918	Brussels Sprout
ELC0802	21/07/2008	Snainton, Yorkshire	SE824918	Cabbage
ELC0803	21/07/2008	Snainton, Yorkshire	SE824918	Red Cabbage
ELC0604P*	11/10/2006	Tayport, Fife	NO457285	Cabbage
ELC0606P*	12/10/2006	Letham, Fife	NO297146	Broccoli

Table 2.2: Code, provider, original collection plant and symbiont infection status for pea aphid lines.
*** indicates pea aphid lines used as positive controls for diagnostic PCR (section 2.2.4.1)**

Code	Provided by	Collection Plant	Symbiont Status
TLW03/01	Angela Douglas	*Medicago sativa*	None
JF99/04	Angela Douglas	*Lotus pedunculatus*	*H. defensa**
JF98/24	Angela Douglas	*Vicia faba*	*R. insecticola**
PS01	Glen Powell	*Vicia faba*	*S. symbiotica*
LL01	Glen Powell	*Medicago sativa*	None
N127	Glen Powell	*Medicago sativa*	*H. defensa*
N116	Glen Powell	*Medicago sativa*	*H. defensa*
JF01/29	Glen Powell	*Lathyrus pratensis*	*S. symbiotica**
SH3	Glen Powell	*Pisum sativum*	*S. symbiotica*

Table 2.3 Details of the cabbage aphid lines used for the experiments in Chapters 2-5, including the number of lines used each time.

			Cabbage Aphid Line									
Chapter	**Experiment**	**No. of Lines**	ELC0603	ELC0604	ELC0605	ELC0606	ELC0607	ELC0608	ELC0610	ELC0611	ELC0612	ELC0613
2	Microscopy	4		X		X	X		X			
2	Diagnostic PCR for 2º symbionts	6	X			X	X		X	X		
2	16S-23S PCR	20	X	X	X	X	X	X	X	X	X	X
2	16S PCR	6				X		X				
2	Cloning and sequencing	5			X	X		X		X		
3	qPCR for Group 1 and 2 bacteria	16		X	X	X	X		X	X	X	X
4	Aphid Glasshouse Exp. 1	3				X	X					
4	Aphid Glasshouse Exp. 2	4		X	X				X			
4	Field Experiment	3			X				X			
4	Parasitism Experiment	2										
5	qPCR for *Buchnera* density	9		X	X	X	X		X	X		

(Cont.)			Cabbage Aphid Line									
Chapter	**Experiment**	**No. of Lines**	ELC0615	ELC0618	ELC0619	ELC0701	ELC0703	ELC0801	ELC0802	ELC0803	ELC0604P	ELC0606P
2	Microscopy	4										
2	Diagnostic PCR for 2º symbionts	6	X									
2	16S-23S PCR	20	X	X	X	X	X	X	X	X	X	X
2	16S PCR	6			X			X	X	X		
2	Cloning and sequencing	5				X						
3	qPCR for Group 1 and 2 bacteria	16		X	X	X	X	X		X	X	X
4	Aphid Glasshouse Exp. 1	3				X						
4	Aphid Glasshouse Exp. 2	4			X							
4	Field Experiment	3			X							
4	Parasitism Experiment	2									X	X
5	qPCR for *Buchnera* density	9			X	X	X					

Plate 2.1: Cabbage aphid clonal lines cultured in stacked plastic cups on excised brussels sprout leaves.

2.2.2 Visualisation of bacteria within aphids using microscopy techniques.

2.2.2.1 Isolation of the bacteria in aphid samples prior to staining.

Samples of approximately 25 aphids from aphid lines ELC0604, ELC0606, ELC0607 and ELC0610 were washed in 1% Tween 20 solution for approximately twenty minutes on a rocker block to remove surface contaminants including bacteria. The Tween 20 solution was removed by pipetting and the samples were rinsed twice in sterile distilled water. A micropestle was used to disrupt the aphid tissue in ice-cold homogenising buffer (50 mM Tris-HCl, pH 7.5, 0.25 M sucrose). To remove large fragments of insect exoskeleton the samples were centrifuged at 380 *g* (Eppendorf Desktop MicroCentrifuge 5424, Eppendorf, UK) for five minutes. The supernatant was transferred to a separate tube and centrifuged at 18,400 *g* for fifteen minutes to pellet the bacterial cells (Darby *et al.* 2001). The supernatant was discarded and the bacterial pellet resuspended by flicking with 100 µl of molecular biology grade (MBG) water.

2.2.2.2 Gram-staining bacteria isolated from aphid samples.

Initially, 50 µl of bacteria that had been isolated in MBG water from aphid tissue (Section 2.2.2.1) was pipetted onto a microscope slide (76x26 mm, VWR International, Leuven). The slide was placed on a heat block at 60ºC for approximately thirty seconds to ensure that the bacteria were firmly mounted to the slide. A few drops of the primary (Gram) stain crystal violet (2 g 90% crystal violet dissolved in 20 ml of 95% ethyl alcohol) were then added and the slide was incubated at 60ºC for one minute. Then a few drops of the mordant Gram's iodine (1 g of iodine, 2 g of potassium iodide, dissolved in 300 ml of distilled water) were added to fix the stain and the slide was incubated at 60ºC for a further minute, after which the slide was destained with a 1:1 solution of ethyl alcohol:acetone. Gram-positive bacteria retained the primary violet stain whereas Gram-negative bacteria were colourless. A secondary stain, safranin 'O' (1 g safranin 'O' dissolved in 10 ml distilled water), was then added and the slide was incubated for one minute at 60ºC then washed with sterile distilled water for a maximum of five seconds. Gram-positive bacteria retained the primary violet stain and did not take up the secondary stain while Gram-negative bacteria took up the secondary stain and appeared red-pink in colour. Slides were

left to air dry before viewing under a Nikon EC-1 confocal microscope (Nikon Instruments Europe B.V., Surrey, U.K.) at x1000 magnification. A sample of *Escherichia coli* (Gram-negative bacteria) was used as a control to ensure the staining procedure had worked efficiently.

2.2.3 DNA extraction from aphids.

DNA was extracted from aphids using a DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, California). The extraction protocol was modified for efficiency from the Qiagen Supplementary Protocol: "Purification of total DNA from insects using the DNeasy® Blood & Tissue Kit (DY14-Aug06)", to include an additional centrifugation step after the lysis stage to prevent aphid exoskeleton from clogging the spin column. Initially aphids were washed in a 500 µl solution of 1% Tween 20 detergent for 20 minutes on a rocking platform to remove any surface contaminants and the waxy outer coating characteristically harboured by *B. brassicae*. The detergent was removed and the samples were rinsed three times in 500 µl of sterile distilled water. The washed aphids were macerated in 2 ml Eppendorf tubes suspended in liquid nitrogen using an ethanol sterilised micropestle.

To the macerated aphid material 180 µl of buffer ATL was added with 20 µl of Proteinase K and the solution was vortexed. Samples were incubated at 55ºC on a heat block (Grant QBTP Heat Block, Grant Instruments, UK) for one hour and vortexed every fifteen minutes. The samples were vortexed again for fifteen seconds, then 200 µl of buffer AL was added before a further vortex and the samples were incubated at 70ºC on a heat block for ten minutes. After ten minutes the samples were centrifuged (Eppendorf Desktop MicroCentrifuge 5424, Eppendorf, UK) for two minutes at 6000 *g* to pellet the debris material, i.e. the hard aphid exoskeleton. The supernatant was transferred to a clean tube containing 200 µl of 100% ethanol and vortexed. Samples were then transferred onto the centre of the membrane of a DNeasy mini spin column, which were placed in 2ml collection tubes and centrifuged at 6000 *g* for one minute. The columns were subsequently washed twice, first with 500 µl of buffer AW1 and centrifuged for one minute at 6000 *g*, then with 500 µl of buffer AW2 and centrifuged for three minutes at 18,500 *g* using a new collection tube each time. After washing, the spin column was placed in a sterile 1.5 ml

Eppendorf and 200 µl of buffer AE was added to the centre of the spin column membrane. The column was incubated at room temperature (22ºC) for one minute, then centrifuged for one minute at 6000 *g* to elute the DNA.

The concentration of the eluted DNA was measured on a Full Spectrum UV/Vis NANODrop Spectrophotometer (ND-1000, Labtech International). Concentrations greater than 50 ng/µl were considered sufficient for PCR amplification. Eluted DNA was then divided into 20 µl aliquots and stored at -20ºC.

2.2.4 PCR screening to determine the presence or absence of bacteria other than **Buchnera** *in cabbage aphid.*

2.2.4.1 Diagnostic PCR amplification for previously characterised aphid secondary symbiont types.

Diagnostic polymerase chain reaction (PCR) assays performed on an Eppendorf Mastercycler® ep gradient PCR machine (Eppendorf AG, Hamburg, Germany) were used to ascertain whether a sub-set of six cabbage aphid lines (ELC0603, ELC0606, ELC0607, ELC0610, ELC0611, ELC0615) possessed any of the secondary symbionts previously characterised in pea aphid (Douglas *et al.* 2006a). Polymerase chain reaction (PCR) is a molecular technique used to amplify a specific region of DNA through a series of denaturation, annealing and elongation steps. DNA extracted from cabbage aphid lines (Section 2.2.3) was used as template for amplification of bacterial 16S rDNA gene fragments using primers developed for three secondary symbiont bacteria characterised in pea aphid. The primers for *S. symbiotica* were the general forward primer 16SA1 (5'-AGAGGTTGATCMTGGCTCAG-3') and the specific reverse primer PASScmp (5'-GCAATGTCTTATTAACACAT-3') (Fukatsu *et al*. 2001). The primers for *H. defensa* and *R. insecticola* were the general reverse primer 16SB1 (5'-TACGGYTACCTTGTTACGACTT-3') with the specific forward primers PABSF (5'-AGCGCAGTTTACTGAGTTCA-3') and U99F (5'-ATCGGGGAGTAGCTTGCTAC-3') respectively (Darby *et al*. 2001; Sandström *et al.* 2001). The PCR reaction mixture contained 1X High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM each dNTP, 1.0 µM each primer, 1.0 Unit High Fidelity Platinum Taq (Invitrogen, UK) and 1 µl DNA extract in a

total volume of 25 µl. The reaction conditions were 30 cycles of 94ºC for 1 minute to denature the DNA, 55ºC for 1 minute to anneal and 72ºC for 2 minutes for elongation, with the denaturing step extended to 5 minutes in the first cycle and the elongation step increased to 8 minutes in the final cycle (Douglas *et al.* 2006a). PCR products were separated by electrophoresis in a 1% agarose gel buffered by 1 x TBE (89 mM Tris-HCl pH 8, 89 mM Boric Acid, 2 mM EDTA) and visualised under ultraviolet light (Alphaimager HP®, Alpha Innotech, California) after staining with SYBR® Safe DNA gel stain (Invitrogen, UK). The sizes of the predicted PCR product for each secondary symbiont, which were differentiated using a 1 kb DNA ladder (Promega, Wisconsin, USA), were approximately 500 bp for *S. symbiotica*, 1660 bp for *H. defensa* and 1500 bp for *R. insecticola* (Darby *et al*. 2001; Fukatsu *et al.* 2001; Sandström *et al.* 2001). DNA template from pea aphid clones known to harbour either, *S. symbiotica*, *H. defensa* or *R. insecticola* was used as positive control for each PCR reaction (Table 2.2).

2.2.4.2 PCR amplification of bacteria other than **Buchnera** *using universal primers.*

PCR amplification was also used to determine the presence or absence of any bacteria other than *Buchnera* in the cabbage aphid lines. For most bacteria other than *Buchnera* the 16S gene is linked to the 23S gene by an Inter Genic Spacer (IGS) region. Non-*Buchnera* sequences can be targeted by amplifying this region using universal primers specific to the two genes and thus can be used to detect the presence of bacteria other than *Buchnera* harboured by aphids (Sandström *et al*. 2001) (Fig. 2.1). A 2.5 kb product spanning the IGS region between the 16S and 23S subunits was amplified from template DNA extracted from the cabbage aphid clonal lines (Section 2.2.3) using an Eppendorf Mastercycler® ep gradient PCR machine (Eppendorf AG, Hamburg, Germany). This method should not produce a PCR product from the few bacteria, such as *Buchnera*, in which the 16S and 23S rRNA genes are not in the same operon (Shigenobu *et al.* 2000; Tamas *et al.* 2002). The primers used were the universal bacterial forward primer 10F 5′-AGTTTGATCATGGCTCAGATTG-3′ (Sandström *et al.* 2001) from the 5’ end of the 16S gene and the universal reverse primer 480R 5’-CACGGTACTGGTTCACTATCGGTC-3’ (Sandström *et al.* 2001) in the 23S gene (Fig.

2.1). In addition the primers 16F27 5'-AGAGTTTGATCCTGGCTCAG-3' (Lane 1991) and 1494R 5'-TACGGYTACCTTGTTACGAC-3' (Lane 1991) on the 16S gene (Fig. 2.1), which amplify from template DNA from all bacteria including *Buchnera*, were included as controls to verify the 16S–23S amplification i.e. negative 16S–23S amplification but positive 16S amplification would increase faith in the ability of the 16S–23S primers to exclude *Buchnera*. The PCR reaction mixture contained 1X High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM each dNTP, 1.0 µM each primer, 1.0 Unit High Fidelity Platinum Taq (Invitrogen, UK) and 1 µl DNA extract in 25 µl total volume. The reaction conditions were an initial denaturation step of 94ºC for 30 seconds then 30 cycles of 94ºC for 1 minute to denature the DNA, 54ºC for 1 minute to anneal, 68ºC for 3 minutes for elongation and then a final elongation step of 72ºC for 10 minutes. PCR products were visualised as described above (Section 2.2.4.1). The expected size for the 16S–23S fragment was 2.5 kb (Sandström *et al.* 2001) and for the 16S only fragment was 1.5 kb. DNA extracted from *Escherichia coli*, a bacterium in which the 16S and 23S genes are within the same operon, was used as a positive control for each PCR reaction.

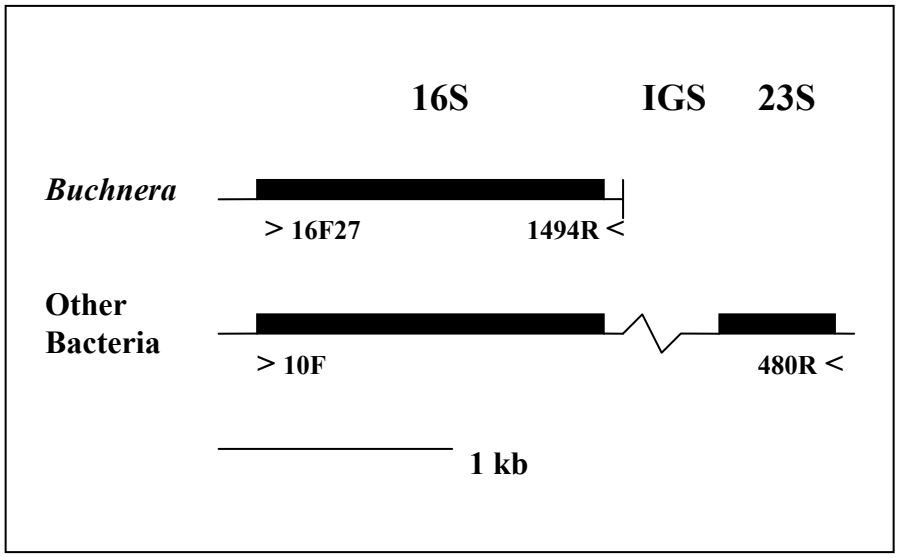


***Figure 2.1: Positions of PCR primers on 16S-23S rDNA genes. (Adapted from Sandström* et al. (*2001*)).**

2.2.5 Characterisation of the bacterial complement in cabbage aphid lines.

2.2.5.1 Molecular cloning and sequencing of bacterial 16S PCR product.

DNA template from cabbage aphid lines was used to generate a PCR product, 2.5kb in length, spanning the IGS region between the 16S and 23S ribosomal subunits with primers 10F and 480R (Section 2.2.4.2). The PCR product was purified using a Minelute PCR Purification kit (Qiagen Inc., Valencia, California) following the manufacturer's instructions and the final concentration of eluted DNA was measured on a NANOdrop spectrophotometer. If the final concentration exceeded 20 ng/µl, the PCR product was cloned with a Strataclone™ Blunt PCR cloning kit (Stratagene, La Jolla, California) following the manufacturer's instructions (Revision #046002). The purified PCR product was ligated into the Strataclone pSC-A-amp PCR cloning vector using a ligation reaction mixture of 3 µl Strataclone cloning buffer, 2 µl of purified PCR product and 1 µl of Strataclone vector mix amp and incubated at room temperature (22°C) for 5 minutes. Heat shock was used to transform 1 µl of the ligation into Strataclone Solopack competent cells by incubation on ice for 20 minutes followed by heat shock in a 42ºC water bath for 45 seconds then incubation on ice for a further 2 minutes. A 250 µl aliquot of SOC buffer (pre-warmed to 42ºC) was added and the tubes were laid horizontally on a shaker at 37ºC with agitation for one hour to allow the competent cells to recover. After one hour, two aliquots (5 µl and 100 µl) of the mixture were plated separately onto LB AIX plates (Amp 100 µg/ml, IPTG 32 µg/ml, X-gal 32 µg/ml) and the plates were incubated overnight at 37ºC.

The Strataclone™ Blunt PCR cloning kit supports a blue/white screening method to determine if the insert has been incorporated in the vector: colonies containing the insert are white while all other colonies are blue. The molecular mechanism for blue/white screening is based on genetic engineering of the *lacZ* operon in the Strataclone competent cells combined with a subunit complementation achieved with the Strataclone vector. The vector encodes the α fragment of the *lacZ* gene with an internal multiple cloning site for PCR product insertion, while the chromosome of the host cell encodes the remaining α subunit to form a functional β-galactosidase enzyme (Sambrook *et al.* 1989). The multiple cloning site can be cleaved by different restriction enzymes so that the PCR product can be inserted within the *lacZ* gene, thus disrupting the production of function of the enzyme β-

galactosidase (Sambrook *et al.* 1989). To determine if the PCR product has been inserted X-gal, a colourless modified galactose sugar that is metabolised by β-galactosidase to form an insoluble product (5-bromo-4-chloroindole) which is bright blue, is used as an indicator (Sambrook *et al.* 1989). The characteristic blue colour of colonies that contain vector without the insert is caused by hydrolysis of colourless X-gal by β-galactosidase. White colonies indicate insertion of the PCR product thus inability of the cell to hydrolyse X-gal (Sambrook *et al.* 1989). The Strataclone vector has topoisomerase-I charged arms, which assist in DNA replication by relaxing and rejoining DNA strands to increase efficiency of insertion of the PCR product into the vector. The Strataclone competent cells express *Cre* recombinase which catalyses recombination between the two *loxP* recognition sites of the Strataclone vector. The combined abilites of topoisomerase-I and *Cre* recombinase are exploited to create a circular DNA molecule (pSC-A-amp/kan) that replicates in cells growing on media containing ampicillin or kanamycin. The resulting pSC-A-amp/kan vector product includes a *lacZ' α*-complementation cassette for blue white screening.

A selection of white colonies were screened for the correct insert (expected size 2.5 kb) using the primers for the insert 10F and 480R and a PCR reaction mix of 5X Green GoTaq reaction buffer (1.5 mM $MgCl_2$), 0.2 mM each dNTP, 10 µM each primer, 1.25 Units GoTaq DNA Polymerase and 2 µl of DNA template (colony picked into 10 µl of water) in a final volume of 25 µl. The reaction conditions were an initial denaturation step of 95ºC two minutes then 30 cycles of 95ºC for 1 minute to denature the DNA, 58ºC for 1 minute to anneal and 72ºC for 2 minutes for elongation and a final elongation step of 72ºC for 5 minutes. PCR products were visualised as above (Section 2.2.4.1). If the desired 2.5 kb insert amplified in a sufficient proportion of colonies (>80%) then individual white colonies were picked into 70 µl of freezing media with Ampicillin (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, K_2HPO_4 6.3 g/l, KH_2PO_4 1.8 g/l, Na citrate 0.45 g/l, $MgSO_4 \cdot 7H_2O$ 0.09 g/l, glycerol 4.4 g/l, Ampicillin 0.15 mg/ml) in each well of a 384 well plate (#7001, GENETIX, New Milton, United Kingdom) and incubated overnight at 37ºC. After 12 hours growth, 5 µl of the freezing media mixture was inoculated into 1 ml 2X LB with Ampicillin (Select peptone 10 g/l, Select yeast 5 g/l, NaCl 5 g/l, Glucose 1 g/l, Select Agar 15 g/l, pH 7.5 with NaOH, Ampicillin 0.15 mg/ml) in each well of a 96 deep well block. The 96 deep well block (AB-0932, ABGene, Epsom, United Kingdom) was then

covered with a gas permeable seal (AB-0718, ABGene, Epsom, United Kingdom) and incubated with shaking for 24 hours at 37ºC (C24KC Refrigerated Incubator Shaker, New Brunswick Scientific, Edison, USA).

The competent cells were pelleted by centrifugation of the block for 5 minutes at 1600 *g* using a Sigma 4K15 Centrifuge (Sigma Laboratories Centrifuges). To prepare the plasmid for sequencing, the pellets were initially resuspended in 80 µl of solution I (0.015 M Tris-HCl pH 8.0, 0.03 M EDTA pH8, 0.03 M Glucose, 0.06 mg/ml RNaseA) and vortexed until they were completely suspended. A volume of 80 µl of solution II (1% SDS, 0.2 M NaOH) was then added to each well and the block was vortexed for one minute. The block was then vortexed again for one minute after 80 µl of solution III (3.6 M KOAc, 14% Glacial Acetic acid) was added. To bind the plasmid to the plate 150 µl of 8 M Guanidine hydrochloride was then added to each well of a Millipore multiscreen binding plate (Millipore, Bedford, USA), which was then placed in the lower part of a vacuum manifold (KNF Laboport, New Jersey, USA). The bacterial lysate in the 96 well block was mixed twice by pipetting, then 220 µl from each well was transferred individually into the corresponding well of a Millipore multiscreen clearing plate (Millipore, Bedford, USA). The clearing plate was then placed on top of the binding plate on the vacuum manifold and the vacuum was applied for three minutes to draw the lysate through the binding plate. The lysate and binding buffer (8 M Guanidine hydrochloride) were mixed three times by repeated pipetting and the vacuum was applied again for one minute. The binding plate was washed twice by adding 200 µl of 100% ethanol to each well then the vacuum was applied for one minute for the first wash and three minutes for the second. The membranes of the binding plate were dried by centrifuging for ten minutes at 1600 *g* and leaving open on the bench for a further ten minutes. The binding plate was then placed on top of a 96 well Microtitre storage plate (Sterilin, United Kingdom), 100 µl of distilled water was applied to each well and the plates were centrifuged at 1600 *g* for five minutes to elute the plasmid. The quality of plasmid product from randomly selected clones was confirmed by gel electrophoresis as described previously (Section 2.2.4.1).

Initially plasmids were sequenced from the 3' end of the 16S subunits using the 10F forward primer (Fig. 2.1). Approximately 600 bp of 16S rDNA sequence was generated by PCR using 3 µl plasmid preparation in a 10 µl final volume of BigDye Version 2.0

(Applied Biosystems, Warrington, UK) sequencing reaction and 10 µM 10F primer. The sequencing reaction conditions were an initial step of 1 minute at 96ºC then 25 cycles of 10 seconds at 96ºC, 5 seconds at 50ºC and 4 minutes at 60ºC. Sequencing reactions were cleaned up by ethanol precipitation. The clean up reaction involved adding 2.5µl 125mM EDTA pH 8.0 then vortexing the plate before adding 30 µl of 95% ethanol to each well. The plate was vortexed then incubated at room temperature (22ºC) for 15 minutes. After incubation the plate was centrifuged at 4ºC for 30 minutes at 1600 *g*. The plate was then inverted and centrifuged for 10 seconds at 100 *g* to remove the ethanol. The plate was washed twice with 150 µl 70% ethanol, each time centrifuging at 4ºC for 10 minutes at 1600 *g* followed by an inverted centrifugation for 10 seconds at 100 *g*. After the final wash the plate was left to air dry for 20 minutes before being analysed on an ABI3700 capillary sequencer (Applied Biosystems, Warrington, UK).

Double strand sequencing of the 16S gene only was performed on randomly selected samples of each identified sequence group (Section 2.2.5.2) using the primers: 954F 5'-GCACAAGCGGTGGAGCATGTGG-3' (Huws *et al.* 2007); 1492R 5'-GGTTACCTTGTTACGACTT-3'(Lane 1991); 533F 5'-GTGCCAGCAGCCGCGGTAA-3' (Huws *et al.* 2007); 1056R 5'-ACACGAGCTGACGACAGCCA-3' (Allen *et al.* 2001) and 522R 5'-GTATTACCGCGGCTG-3' (Allen *et al.* 2001) (Fig. 2.2). The procedure was the same as described for the 10F primer sequencing described above.

2.2.5.2 Molecular identification of bacterial sequence from cabbage aphid lines.

To group related sequences a maximum likelihood phylogenetic tree (RAxML (randomised axelerated maximum likelihood) + Cat model) with 100 bootstraps based on the partial 16S rDNA sequence generated using the 10F primer was drawn in TOPALi Version 2 (Milne *et al.* 2004). The partial 16S sequence for each clone was grouped within a sequence type according to particularly robust bootstrap values and long-branch lengths at a rate of 0.01 substitutions per base level. If a clone appeared to represent a rare sequence type and the reaction was poor re-sequencing was performed to ensure that the sequence was of sufficient quality. If phylogenetic analysis was based only on the first 600 bp then substantial amounts of information could have been lost (Griffiths *et al.* 2006). Consequently, double strand sequence was generated for at least eight randomly selected

examples of each sequence type. Where less than eight examples were available for a sequence group all sequences were analysed. Full length 16S sequence (~1500 bp) for both strands was generated for each clone using six different primers (3 forward and 3 reverse) (Fig. 2.2) to generate sufficient overlapping segments of sequence and obtain a reliable consensus sequence for each clone by manual editing. Raw sequence data was quality scored using *Phred* (Ewing and Green 1998; Ewing *et al*. 1998) and contigs were assembled and the consensus sequence was edited for each clone using Sequencher software (Gene Codes Corporation, Michigan, USA) for each clone (Fig. 2.2). Although the IGS and 23S subunits were amplified they were not sequenced as it was expected that the 16S subunit alone would provide sufficient information for characterisation.

Consensus sequences for each 16S contig were aligned using the partial order alignment program POA (Lee *et al.* 2002) and inspected, with adjustments made to optimise the alignment, in Sequencher (Gene Codes Corporation, Michigan, USA). A maximum likelihood tree (RAxML + Cat Model) with 100 bootstraps was drawn based on the consensus sequences to group sequence types more accurately according to the full length of the 16S sequence. Up to eight examples of consensus sequence from each group were then BLAST searched against the NCBI sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the bacterial order to which each belonged and their similarity to other bacterial 16S sequence. The similarity threshold reported by BLAST was used to determine the similarity of cabbage aphid 16S bacterial sequence types to 16S sequence of other characterised bacteria in the NCBI database. The minimum accepted similarity threshold for species definition is 97% i.e. a level of sequence divergence between species of >3% (Stackebrandt and Goebel 1994).

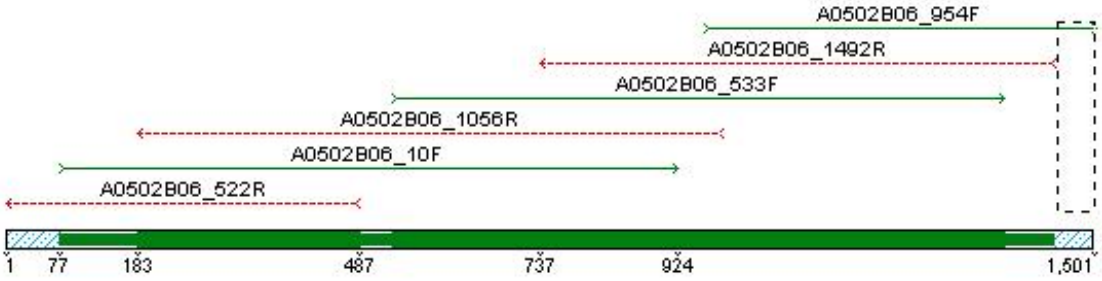


Figure 2.2: Example contig showing the position and direction of all six sequencing primers on the 16S subunit.

2.2.5.3 Characterisation of cabbage aphid bacterial sequence types using phylogenetic analysis.

To further characterise the bacteria found in cabbage aphid their phylogenetic relationship with other types of bacteria was determined by generating a representative consensus sequence for each sequence type using five example contigs (Section 2.2.5.2). Whenever five were not available then all available examples were used. Although within group sequence variation was low, small differences in the sequence of each clone could reduce the accuracy of phylogenetic analysis (Naum *et al.* 2008), which could be avoided by generating a representative sequence for each group of sequences. The representative sequences for each sequence type were named 'Cabbage Aphid Type' and numbered for identification. The accession numbers of 16S sequence from published 16S phylogenies of the bacterial orders, to which the cabbage aphid sequences had been assigned according to their similarity to database sequences, were used to collate database sequences from the NCBI database. Failure of the 16S gene to identify sequences to the species level has been attributed partly to high proportions (>1%) of undetermined nucleotide bases in database 16SrDNA sequences (Drancourt *et al.* 2000). Consequently, the quality of all sequences was checked before inclusion in any phylogenetic analysis. The cabbage aphid representative sequence types and the database sequences were aligned separately for each bacterial order using POA. Based on the alignment, a separate maximum likelihood phylogenetic tree (RAxML + Cat Model) with 100 bootstraps was drawn for each taxonomic order of bacteria.

2.2.5.4 Accurate representation of the diversity of cabbage aphid sequence types was essential.

Three additional steps were included in the sequence analysis to ensure that the study captured an accurate representation of the diversity of bacterial sequence types found in cabbage aphid. Firstly, separate trees were generated from the 5' and 3' halves of the alignment to check for potential chimeric sequences resulting from annealing of non-target during PCR amplification or recombinant DNA during vector cloning (Ashelford *et al.* 2005). Drawing separate trees for both halves of the alignment allowed visual identification of chimeric sequences that exhibited variation in their position according to which half of

the tree was analysed. Candidate chimeric sequences were then analysed using Sequencher to identify chimers, which were removed from further analyses. It is important to identify all possible chimeric sequences because they can cause inaccurate phylogenetic tree construction and recent estimates have suggested that at least 1 in 20 16S rRNA bacterial sequence records held in the databases contain chimeric abnormalities (Ashelford *et al.* 2005). Secondly, rarefaction analysis, with and without single clone types (i.e. sequence types which were represented by only one clone) was used to test whether sequencing of clones had been sufficient to give an exhaustive representation of all possible sequence types (Gotelli and Entsminger 2007; http://garyentsminger.com/ecosim.htm). Rarefaction is an ecological technique used to standardise and compare species diversity according to different sample sizes via the construction of species accumulation curves (Gotelli and Colwell 2001). Rarefaction can be applied to investigate saturation of clone libraries by considering sequence types as species. The accumulation curve is a plot of the number of sequence types as a function of the number of clones sequenced, and the slope of the curve determines the frequency of any additional sampling required (Gotelli and Colwell 2001). If additional sampling will only yield a small number of new types the curve will flatten or 'saturate' and if a large number of types still remain to be sequenced the curve will increase linearly. Consequently, only when the rarefaction curve of cabbage aphid sequence types saturated could sequencing be considered exhaustive. Thirdly, the diversity of cabbage aphid sequence types detected in this study could be influenced by preferential amplification of dominant amplicons from common bacterial types suppressing the amplification of rare types in complex samples causing what is termed as PCR bias (Becker *et al.* 2000). To determine the effect of PCR bias on the frequency of cloned sequence types in this study PCR product generated using two different 16S–23S primer combinations was cloned and sequenced to compare the resultant frequency clones of each different sequence types. The two additional primer combinations were 16F27/480R and 10F/LD Bact 132 a 18 (5'- CCGGGTTTCCCCATTCGG-3'; Ranjard *et al.* 2001). If all primer combinations yielded a similar frequency of each sequence type to the 10F/480R primer combination then it was assumed the effect of PCR bias was minimal.

2.3 Results

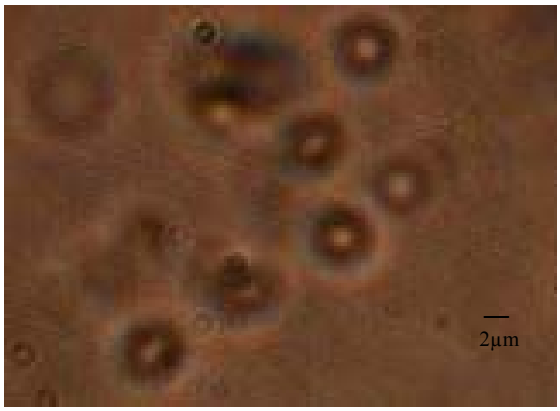
2.3.1 Gram-staining indicated there were both rod-shaped Gram-negative bacteria and **Buchnera** *cocci in cabbage aphid.*

Preliminary Gram-staining indicated that cabbage aphid harboured both large *Buchnera* like cocci (~2 μm in diameter) (Plate 2.2A) and smaller Gram-negative rod-shaped free-living bacteria (~1 μm in diameter) (Plate 2.2B). The *Buchnera* cocci were present in large numbers (~250/aphid) while the rod-shaped bacteria were only detected at low densities (~20/aphid) in two of the four aphid lines investigated (ELC0604 and ELC0606). No Gram-positive bacteria were detected in any of the four cabbage aphid lines indicating minimal contribution of Gram-positive bacteria to the bacterial community of cabbage aphid.

2.3.2 PCR amplification of bacteria in cabbage aphid.

The secondary symbionts *H. defensa*, *R. insecticola* and *S. symbiotica* were not detected by diagnostic PCR in any of the cabbage aphid lines (Douglas *et al.* 2006a). However, a 2.5 kb PCR product was obtained from the 16S–23S ribosomal region in 14 out of the 20 cabbage aphid lines tested (Plates 2.3A&B) indicating clonal variation in the presence of other secondary bacteria. Presence of the 1.5 kb 16S product in all the subset of aphid lines tested (Plate 2.3 B) verified the 16S–23S amplification results and indicated that lack of a 16S–23S product in some aphid lines was not due to inhibition of the PCR assay by the template.

A



B

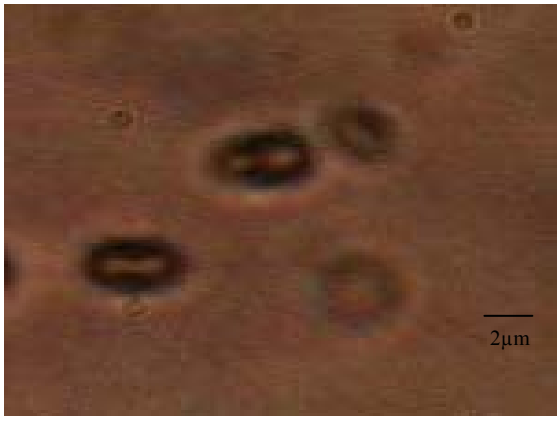


Plate 2.2: Images of bacteria similar in shape and size to **Buchnera** ***cocci (A) and free living rod-shaped bacteria (B) at x1000 magnification.***

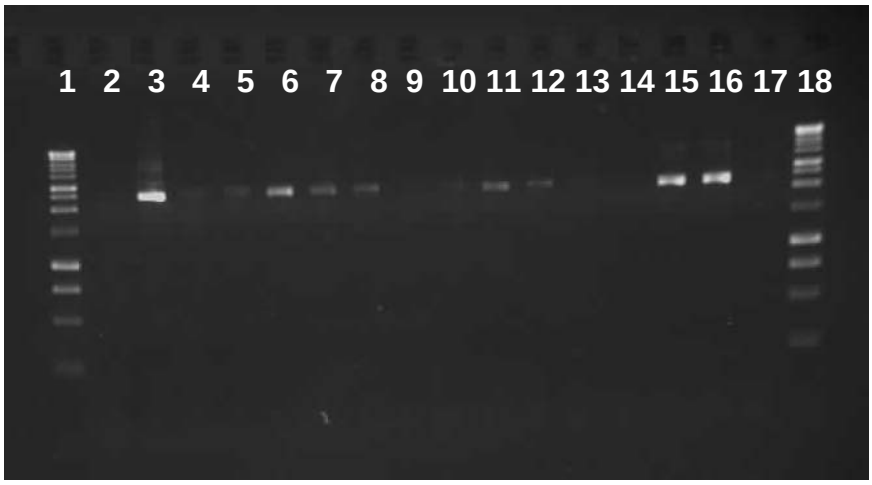


Plate 2.3A: 16S–23S PCR assays for cabbage aphid lines. Negative control MBG H_2O (lane 2), positive control* E. coli *(lane 3), cabbage aphid lines (lanes 4-17). ELC0703 (lane 4), ELC0701 (lane 5), ELC0612 (lane 6), ELC0613 (lane 7), ELC0615 (lane 8), ELC0610 (lane 9), ELC0618 (lane 10), ELC0611 (lane 11), ELC0606P (lane 12), ELC0604P (lane 13), ELC0607 (lane 14), ELC0605 (lane 15), ELC0604 (lane 16), ELC0603 (lane 17). The molecular ladder (lanes 1 and 18) was the Promega 1 kb ladder (Promega, UK) comprising band sizes at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp.

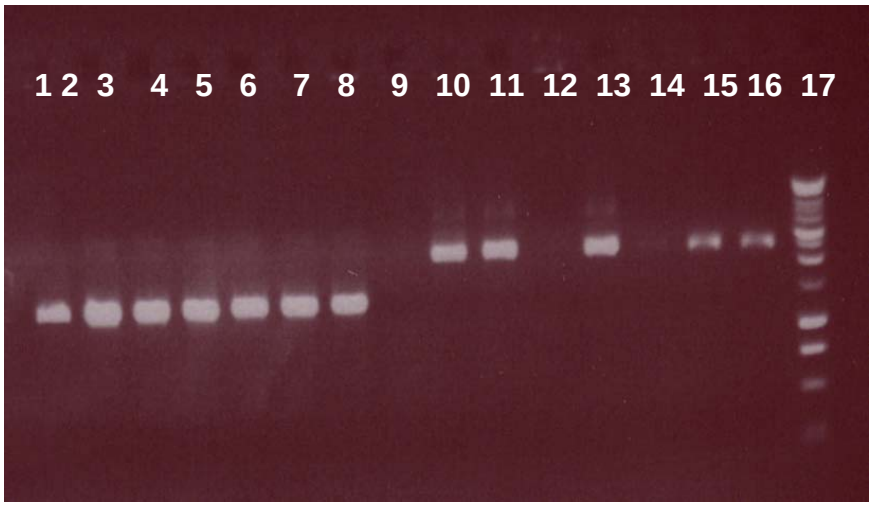


Plate 2.3B: 16S (lanes 1-9) PCR assay for sub-set of cabbage aphid lines and 16S-23S PCR assays for five additional cabbage aphid lines. Negative control MBG H_2O (lanes 1 and 9), positive control* E. coli *(lanes 2 and 10), cabbage aphid lines (lanes 3-8 and 11-16). ELC0619 (lanes 3 and 11), ELC0803 (lanes 4 and 12), ELC0801 (lanes 5 and 13), ELC0802 (lanes 6 and 14), ELC0608 (lanes 7 and 15) and ELC0606 (lanes 8 and 16). The molecular ladder (lane 17) was the Promega 1 kb ladder (Promega, UK) with band sizes same as above.

2.3.3 Sequencing of the 16S gene revealed a wide diversity of bacteria associated with the cabbage aphid.

The 2.5kb 16S-23S PCR product was cloned and the 16S region only was sequenced from five different aphid lines (ELC0605, ELC0606, ELC0608, ELC0611 and ELC0701) chosen at random to characterise the diversity of secondary bacteria associated with the cabbage aphid. Phylogenetic analysis of initial partial length sequences of all clones indicated that the 16S sequence could be grouped into twenty-two different types. However, phylogenetic analysis of consensus sequences generated from full-length 16S sequence (Section 2.2.5.2) reduced the number of groups to fifteen (Fig. 2.3). The frequency of clones of each sequence type varied across the aphid lines indicating that each aphid line harboured a different bacterial complement (Table 2.4). Some sequence types occurred only once (Types 1, 2 and 3), while others were common (Types 7, 14 and 15) (Table 2.4). Molecular comparison with 16S database sequence revealed that the bacterial sequence types cloned from cabbage aphid were all >98% identical to bacteria within five different orders. The majority of sequenced clones belonged to the Pseudomonales and Enterobacteriales. The phylogeny was split into two large clusters reflecting these two orders: Group 1 '*Pseudomonas'* types (8, 9 and 10) and Group 2 '*Erwinia'* types (12, 13, 14 and 15) (Fig. 2.3).

The three different primer combinations used to check for PCR amplification bias in the frequency of cloned cabbage aphid bacterial sequences yielded very similar results (Table 2.5) indicating that there was little evidence for PCR bias. Rarefaction analysis based on all 16S preliminary sequences revealed that when the rare types were present, the rarefaction curve continued to rise and sequencing could not be considered exhaustive (Fig. 2.4). However, when the single rare types were removed from the analysis, the rarefaction curve saturated suggesting a representative sample of the diversity of sequence types had been captured by the clone library. Removal of the singleton sequence types could be justified due to their rarity in cabbage aphid lines and the likelihood that any additional rare types that might be detected by further sampling would have a minimal effect on cabbage aphid fitness in comparison with the common types. For the purposes of this study the fifteen different groups of sequence types detected were considered a representative sample of the diversity of bacteria associated with the cabbage aphid.

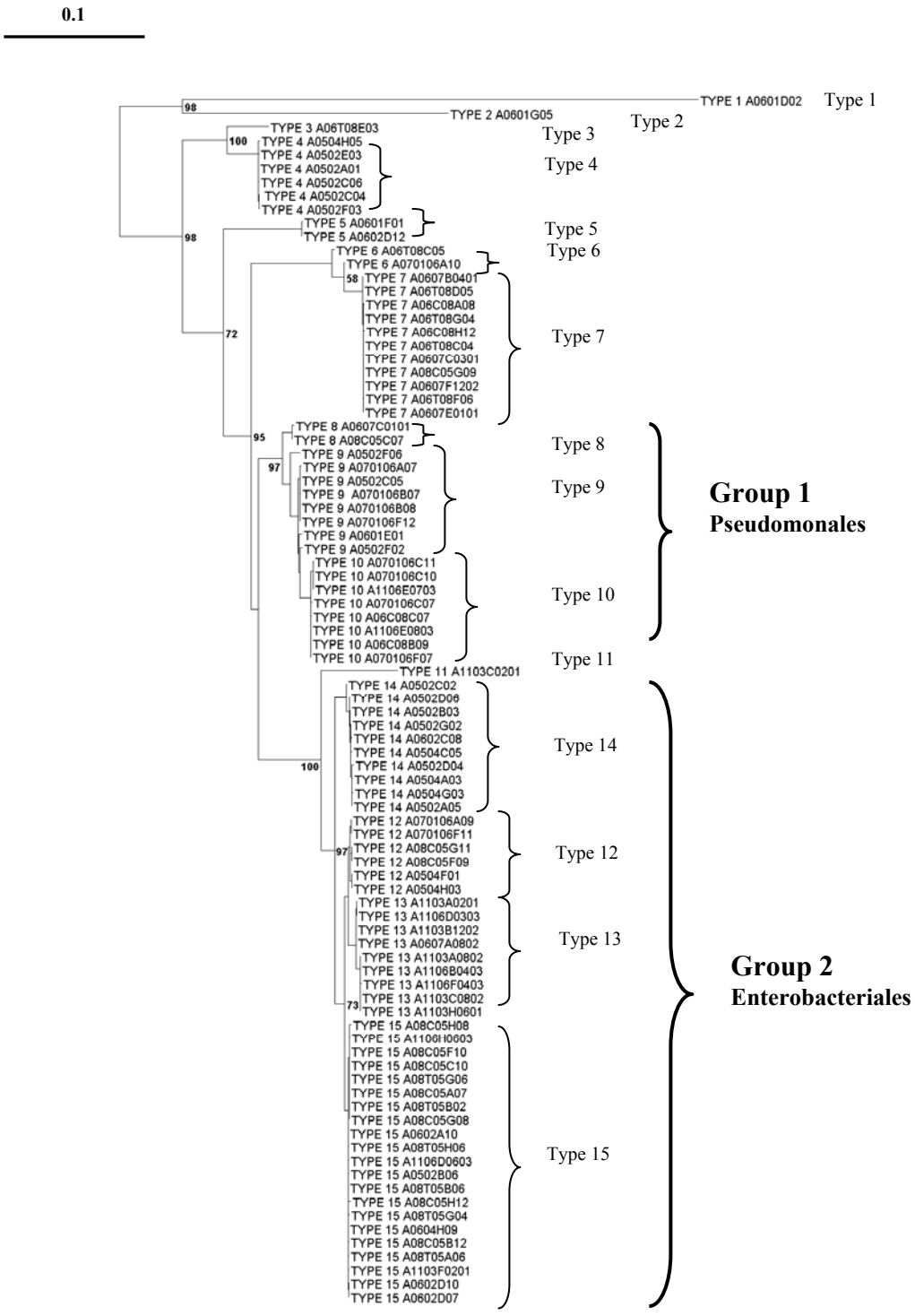


Figure 2.3: Maximum likelihood phylogenetic tree (RAxML+Cat model) based on 16S consensus sequence of clones sequenced in five different cabbage aphid lines indicating fifteen groups of sequence types and the two predominant bacterial orders to which they were assigned according to similarity to bacterial sequence in the NCBI database (scale bar is 0.1 substitutions per base).

Table 2.4: Frequency of cabbage aphid sequence types cloned from DNA extracted from five different cabbage aphid lines.

Cabbage Aphid Type	Aphid Line ELC0606	ELC0605	ELC0611	ELC0608	ELC0701	No. of clones of each type
1	1	0	0	0	0	**1**
2	1	0	0	0	0	**1**
3	1	0	0	0	0	**1**
4	0	8	0	0	0	**8**
5	2	0	0	0	0	**2**
6	1	0	0	0	1	**2**
7	112	0	0	1	0	**113**
8	1	0	0	1	0	**2**
9	4	5	0	1	17	**27**
10	3	0	2	0	2	**7**
11	0	0	1	0	0	**1**
12	0	2	0	2	2	**6**
13	1	0	11	0	0	**12**
14	4	33	2	0	0	**39**
15	107	1	104	88	4	**304**
Total No. of clones sequenced	**238**	**49**	**120**	**93**	**26**	**526**

Table 2.5: Frequency of cabbage aphid sequence types cloned from DNA extracted from cabbage aphid line ELC0606 using three different primer combinations to test for PCR bias.

Cabbage Aphid Sequence Type	Primer Combination 10F/480R	10F/LD Bact 132 a 18	16F27/480R
1	0	0	1
2	0	0	1
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	1
7	0	0	0
8	2	0	0
9	3	4	7
10	0	0	0
11	0	0	0
12	0	1	0
13	0	1	0
14	38	36	36
15	3	4	0
Total No. of clones sequenced	**46**	**46**	**46**

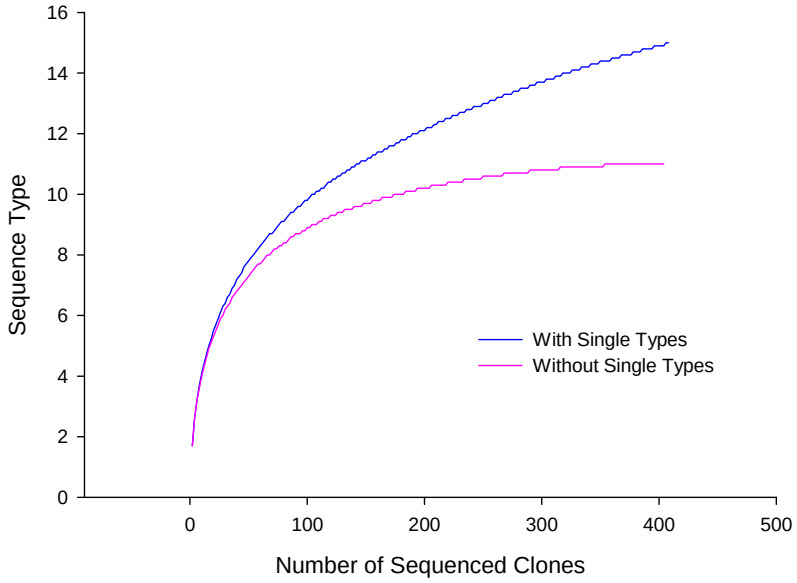


Figure 2.4: Rarefaction analysis of bacterial sequence types with and without removal of bacterial types represented by a single cloned sequence.

Robust bootstrap values (>70) indicated good phylogenetic separation between the different groups of cabbage aphid sequence types (Fig. 2.3) and that the same groups would have been generated at least seventy times out of a hundred independent analyses. Tight clustering of each group of sequences and the short branch lengths of nodes in the tree (Fig. 2.3) indicated low within group variation in 16S sequence. Robust separation between groups and low within group variation meant that the different cabbage aphid sequence types could be characterised separately based on a representative cabbage aphid sequence type generated for each group of sequences (Section 2.2.5.3).

Phylogenetic analysis of 16S sequence consensus sequence and molecular comparison with database sequences revealed that the diversity of cabbage aphid bacterial sequence types spanned five different bacterial orders, the Bacteroidetes, Xanthomonales, Burkholderiales, Pseudomonales and Enterobacteriales (Fig. 2.5). Fourteen of the fifteen different cabbage sequence types grouped within distinct bacterial orders with the exception of cabbage aphid type 2 which matched only sequences from unidentifiable soil bacteria and was therefore omitted from further analyses. The bacterial orders represented by cabbage aphid sequence types included insect symbionts (Burkholderiales, Bacteroidetes and Enterobacteriales) and several examples of insect and plant pathogens (Pseudomonales and Enterobacteriales). The phylogenetic position of each representative cabbage aphid sequence type within each order of bacteria was examined in more detail (Figs. 2.6–2.10) in an attempt to elucidate bacterial function.

The order Bacteroidetes includes the primary endosymbiont of cicadas and sharpshooters *Sulcia muelleri*, the cockroach symbiont *Blattabacterium* and a male killing symbiont found in ladybird beetles. However, cabbage aphid sequence type 1 did not group closely with any of these bacteria and was more closely related to free-living *Flavobacterium* species, and therefore unlikely to exhibit a symbiotic lifestyle (Fig. 2.6). Cabbage aphid sequence type 5 was allied to the order Xanthomonales where it grouped separately from other *Stenotrophomonas* species (bootstrap separation = 99%) indicating that it was not closely related to *Stenotrophomonas* species included in the phylogeny (Fig. 2.7). In the order Burkholderiales cabbage aphid sequence types 3 and 4 were more closely

related to the free living bacteria, *Janthinobacterium* and *Burkholderia* (respectively) than the mealy bug β-symbiont (Fig. 2.8). Within the order Pseudomonales cabbage aphid sequence types 6 and 7 clustered within the *Acinetobacter* species sub-group (Fig. 2.9). Interestingly, the majority of clones of types 6 and 7 occurred in one sample of DNA cloned and sequenced from aphid line ELC0606 (Table 2.3), from which previous samples had yielded only small numbers of type 6 and 7 clones. *Acinetobacter* species are free-living bacteria commonly found in water and soil and therefore could be environmental contaminants, particularly given that the majority occurred in one sample of extracted aphid DNA. In order to minimise the presence of contaminant bacteria in aphid samples, aphids were washed prior to DNA extraction in Tween 20. A comparison of cloning and sequencing results from Tween 20 versus non-Tween 20 washed aphids revealed that after Tween 20 washing the number of clones assigned to *Acinetobacter* species was greatly reduced. Consequently, cabbage aphid types 6 and 7 were likely to be environmental contaminants which were either gut 'tourists' picked up by the aphid or were not excluded by the washing process in one sample of ELC0606 aphids.

Cabbage aphid types 8, 9 and 10 were allied to the order *Pseudomonales* and grouped closely with two different groups of *Pseudomonas* species defined by Anzai *et al.* (2000). Cabbage aphid sequence types 8 and 9 were found within the "*Pseudomonas aeruginosa*" group while cabbage aphid type 10 was found in the "*Pseudomonas fluorescens"* group (Fig. 2.9). Cabbage aphid type 8 grouped closely with *Pseudomonas aeruginosa,* which is an opportunistic pathogen of insects including *Drosophila* (D'Argenio *et al.* 2001). The order Pseudomonales includes many pathogenic bacteria, which could influence cabbage aphid fitness but does not include any bacteria known to form symbiotic relationships with insects.

Although many sequenced 16S clones fell within the order Pseudomonales the largest proportion of sequenced clones were assigned to the order Enterobacteriales (Table 2.4). The order Enterobacteriales contains numerous examples of insect symbiotic bacteria including *Buchnera* and the aphid secondary symbionts, *R. insecticola*, *H. defensa* and *S. symbiotica*. Within the order Enterobacteriales the cabbage aphid sequence types (11–15) were found within different groups of the family Enterobacteriaceae. Cabbage aphid type 11 was highly similar to *Buchnera* in pea aphid (*A. pisum*) (Fig. 2.10) indicating that a

single cloned sequence of the cabbage aphid primary symbiont *Buchnera* had been amplified non-specifically in the 16S–23S PCR amplification (Table 2.4). Of the remaining four types of cabbage aphid sequence all grouped more closely to free-living rather than symbiotic bacteria (Fig. 2.10). Cabbage aphid types 12 and 15 grouped closely with *Erwinia amylovora* and other *Erwinia* species within the phylogenetic sub-group of *Erwinia* species defined as 'Cluster I' by Brown *et al.* (2000) (Fig. 2.10). *Erwinia* species within 'Cluster I' were all necrogenic bacteria that cause cell death leading to lesions and extensive tissue death (Brown *et al.* 2000) indicating that the cabbage aphid types 12 and 15 could have similar capabilities. Similarly, cabbage aphid type 14 grouped closely with *Pectobacterium carotovora* in 'Cluster II', a sub-group of *Erwinia* species with soft-rotting capabilities (Brown *et al.* 2000) (Fig. 2.10). Cabbage aphid type 13 did not group within the *Erwinia* clusters but was found within the *Enterobacter* species group of the Enterobacteriaceae family (Fig. 2.10). Interestingly, cabbage aphid sequence type 13 grouped very closely with *Erwinia aphidicola*, which has been isolated from the gut of pea aphid and shown to reduce pea aphid growth and fecundity (Harada *et al.* 1997; Harada and Ishikawa 1997). The grouping of *Erwinia aphidicola* and cabbage aphid type 13 with *Enterobacter* species rather than the other *Erwinia* species (Fig. 2.10) suggests that these bacteria may differ from plant pathogenic *Erwinia* species and potentially might represent a novel clade of bacteria within the Enterobacteriaceae. The bacteria associated with cabbage aphid thus exhibited some degree of taxonomic affiliation with known insect symbiotic bacteria and pathogens.

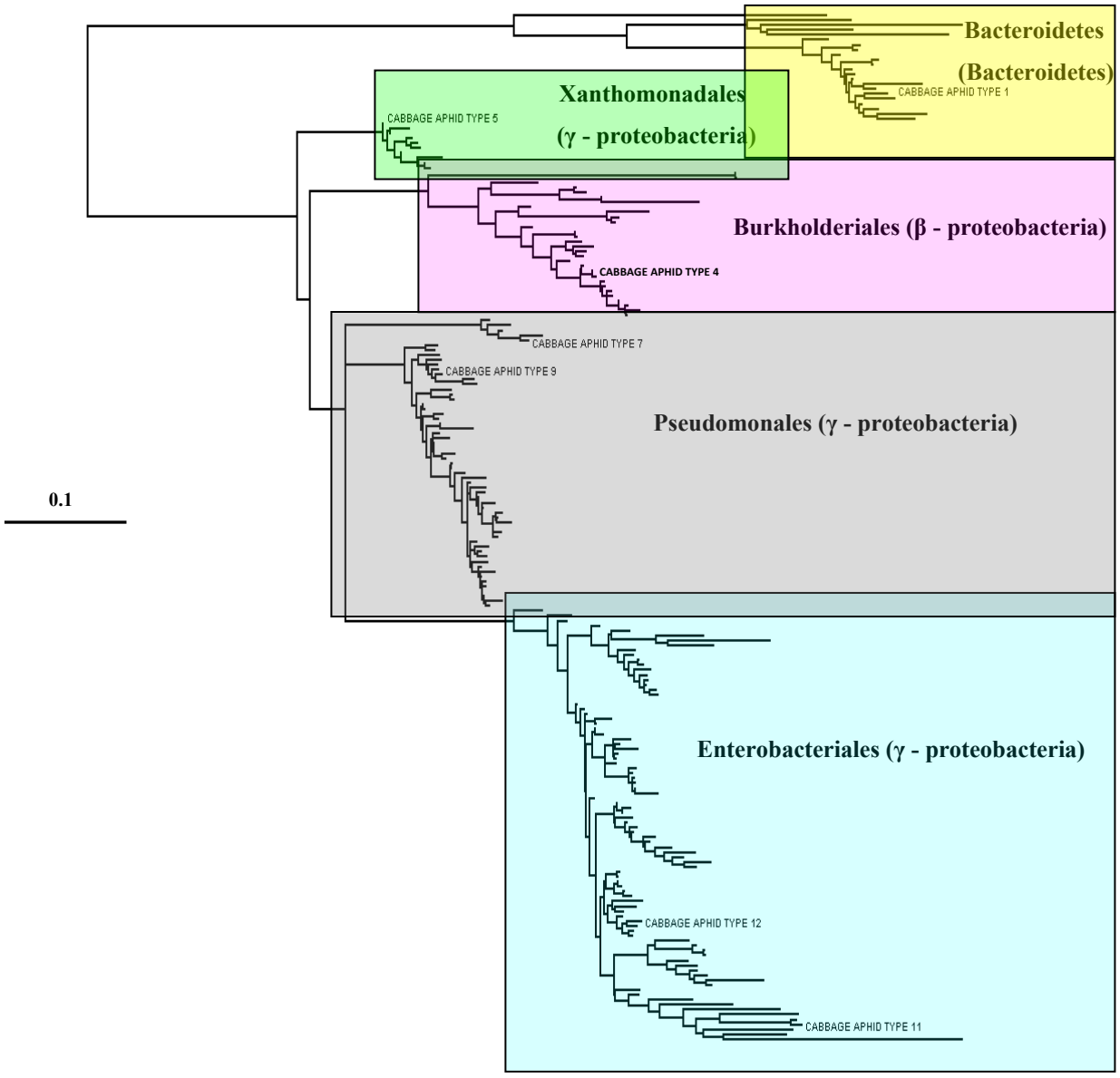


Figure 2.5: Maximum likelihood skeletonised tree (RAxML + Cat Model) based on 16S bacterial database sequences for each class or order of bacteria together with sequences discovered in this study to illustrate the overall distribution of bacterial orders containing bacterial sequence types derived from cabbage aphid (scale bar is 0.1 substitutions per base).

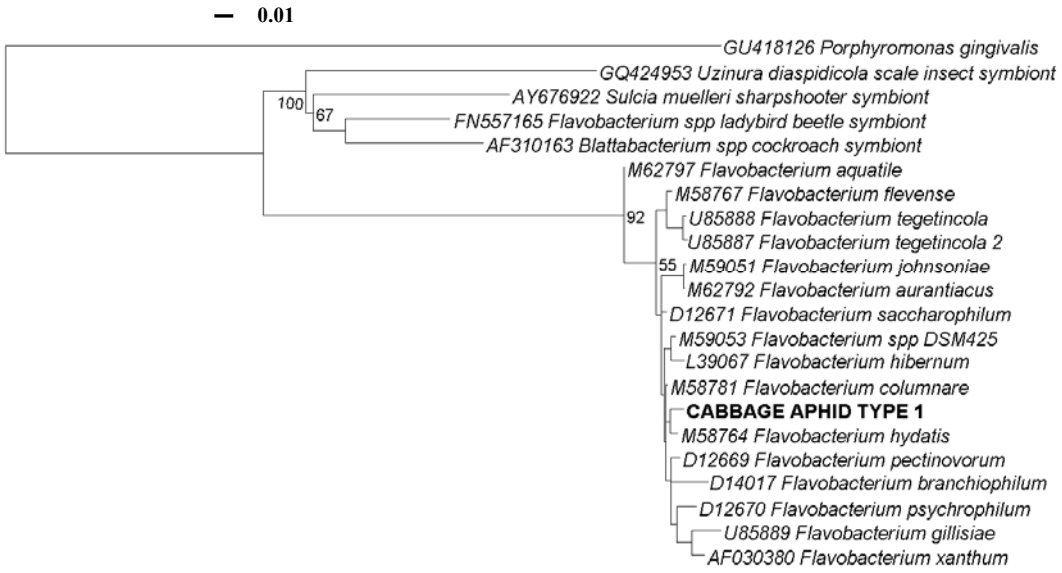


Figure 2.6: Maximum likelihood phylogenetic tree (RAxML + Cat model) to illustrate the position of cabbage aphid bacterial sequence types within the class Bacteroidetes (16S phylogeny from McCammon and Bowman (2000) including additional insect symbiont sequences in the Bacteroidetes) (scale bar is 0.01 substitutions per base).

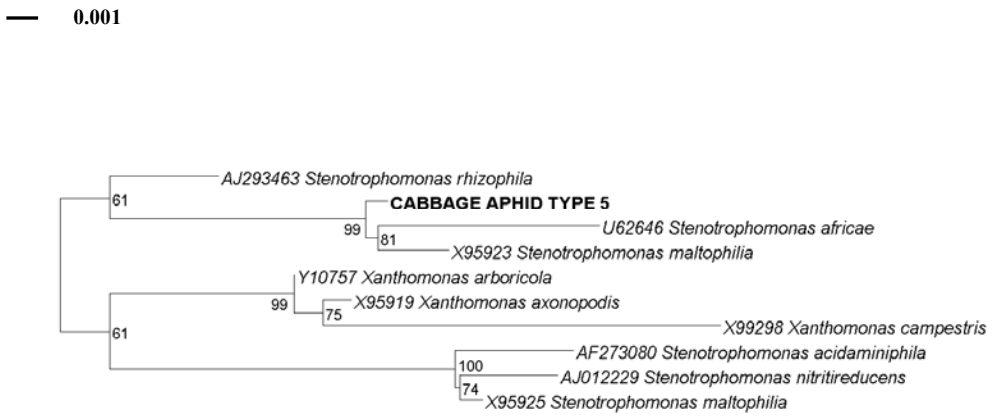


Figure 2.7: Maximum likelihood phylogenetic tree (RAxML + Cat model) to illustrate the position of cabbage aphid bacterial sequence types within the order Xanthomonadales (16S phylogeny from Assih* et al. *(2002)) (scale bar is 0.001 substitutions per base).

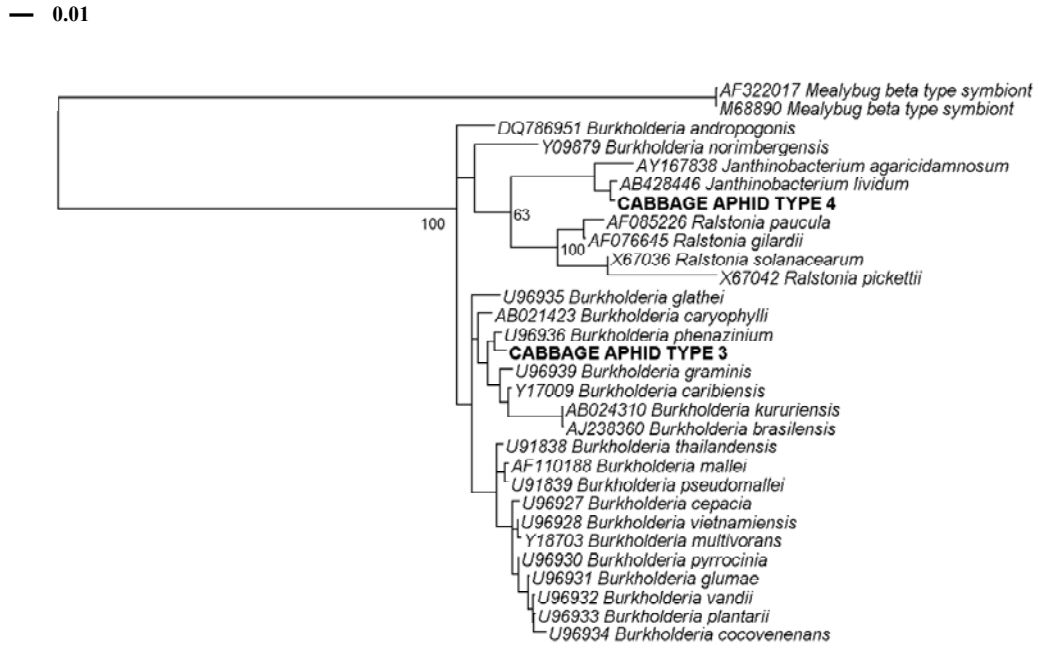


Figure 2.8: Maximum likelihood phylogenetic trees (RAxML + Cat model) to illustrate the position of cabbage aphid bacterial sequence types within the order Burkholderiales (16S phylogeny from Fain and Haddock (2001) including additional insect symbiont sequences in the Burkholderiales) (scale bar is 0.01 substitutions per base).

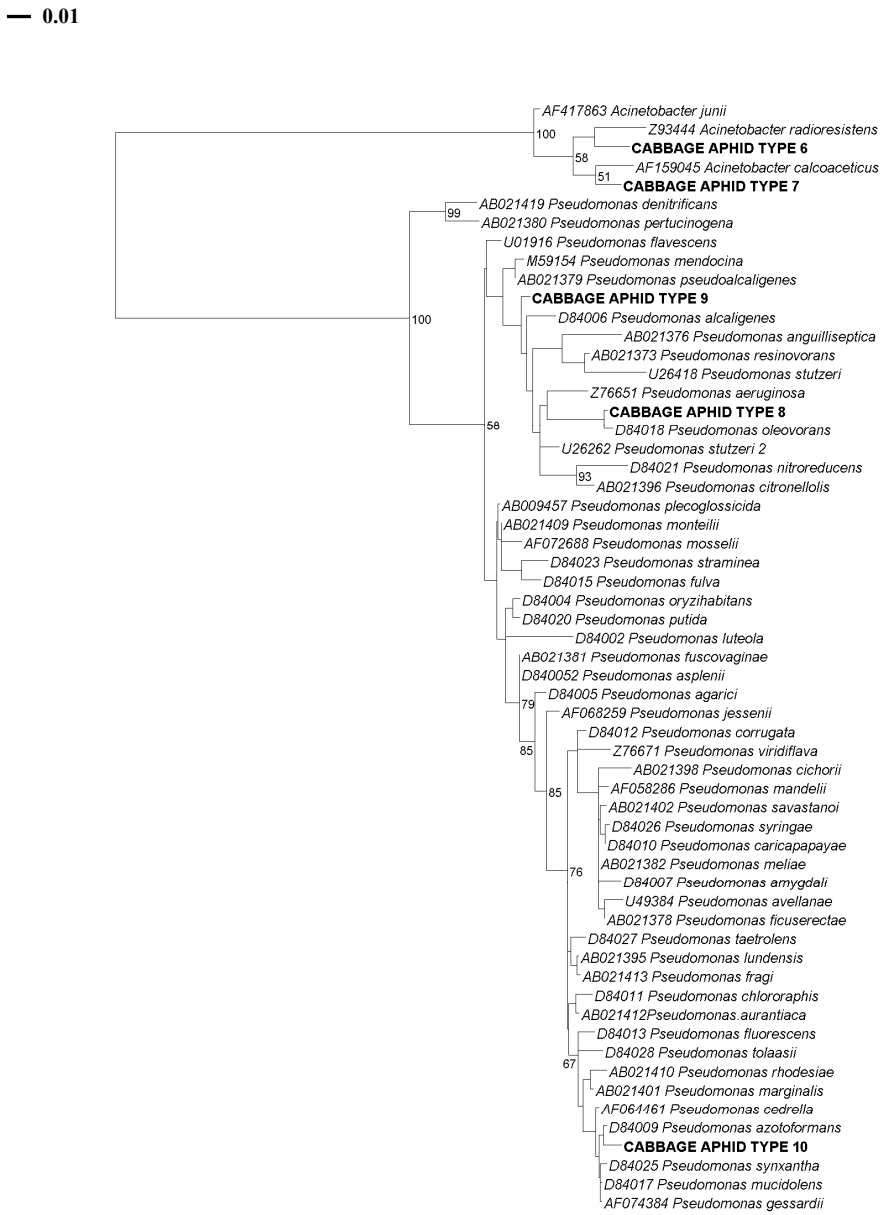


Figure 2.9: Maximum likelihood phylogenetic tree (RAxML + Cat model) to illustrate the position of cabbage aphid bacterial sequence types within the Pseudomonales (16S phylogeny from Anzai* et al. *(2000)) (scale bar is 0.01 substitutions per base).

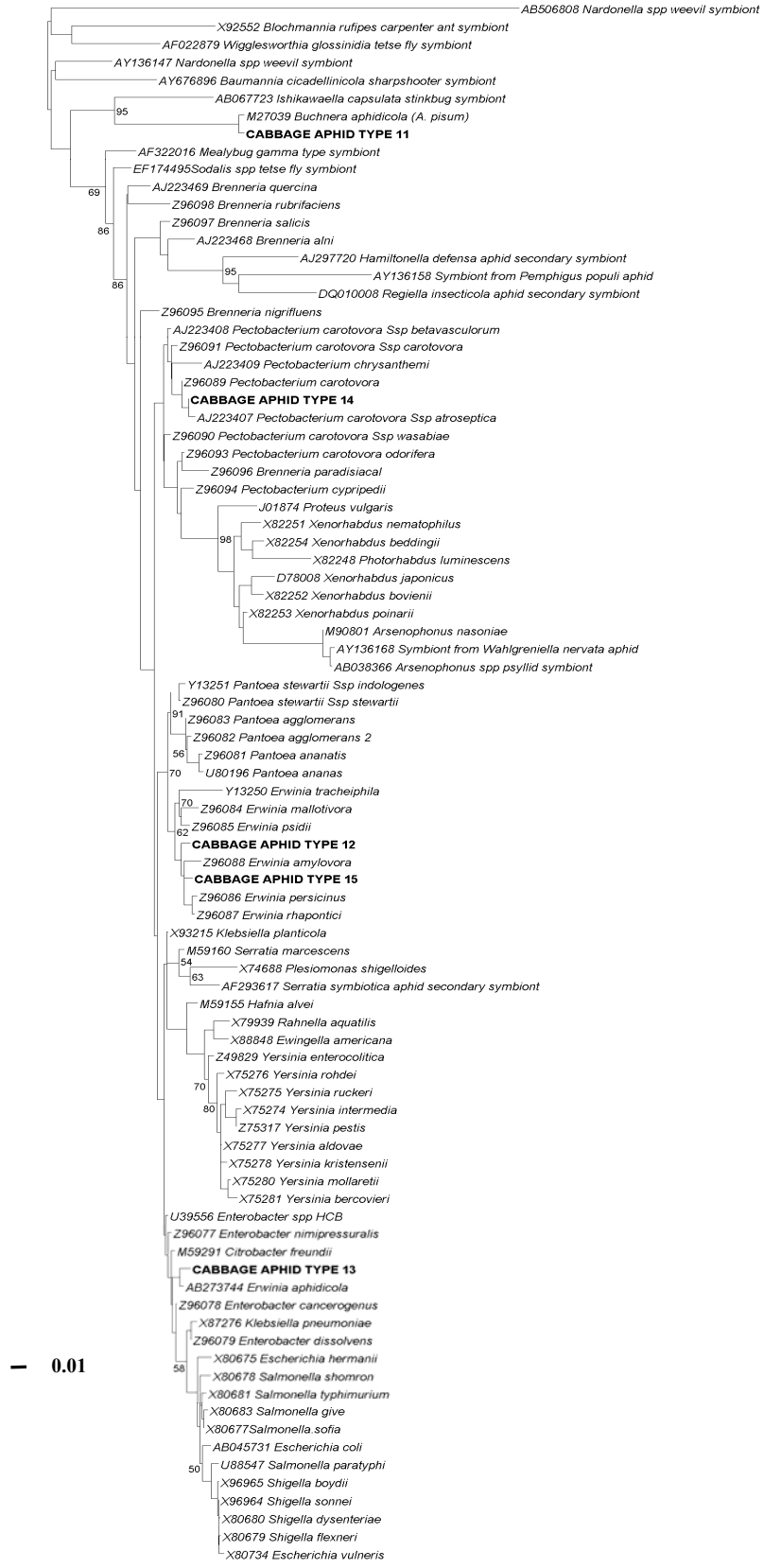


Figure 2.10: Maximum likelihood phylogenetic tree (RAxML + Cat model) to illustrate the position of cabbage aphid sequence types within the order Enterobacteriales. (16S phylogeny from Hauben* et al. *(1998) including additional insect symbiont sequences in the Enterobacteriales) (scale bar is 0.01 substitutions per base).

2.4 Discussion

2.4.1 Characterisation of the bacterial complement of cabbage aphid revealed a diverse community probably associated with the aphid gut.

The diverse bacterial community associated with the cabbage aphid did not include representatives of the secondary symbiont types commonly studied in the pea aphid. Indeed the cabbage aphid bacterial sequence types showed little similarity to any other bacterial symbionts characterised in insects and were more closely related to free-living bacteria. The secondary symbionts of pea aphid are intracellular symbionts, located in specialised structures within the haemocoel (Hinde 1971; Fukatsu *et al.* 2000; Sandström *et al.* 2001) that are maintained in pea aphid genotypes by vertical transmission with occasional horizontal transmission between genotypes (Darby and Douglas 2003; Russell *et al.* 2003). In contrast it is likely that the bacteria found in cabbage aphid rely predominantly on horizontal rarther than vertical transmission and are therefore not subject to the same genetic effects and selective pressures as the pea aphid secondary symbionts. The bacterial 16S sequence is particularly highly conserved among intracellular symbiont bacteria, exhibiting little variation across insect taxa (Darby *et al.* 2001; Moran 2003; Moran *et al.* 2008; Chiel *et al.* 2009). In contrast, the bacterial 16S sequence found in cabbage aphid varied between and within different cabbage aphid clonal lines suggesting that they do not represent intracellular symbionts. It is likely that the bacteria characterised in the cabbage aphid in this study are extracellular, probably associated with the gut for transient or prolonged periods. Other studies have suggested that aphids harbour a limited gut microflora (Harada and Ishikawa 1993; Grenier *et al.* 1994; Harada *et al.* 1996; Harada and Ishikawa 1997) including *Pseudomonas* and *Erwinia* species in the gut of pea aphids (Grenier *et al.* 1994; Harada *et al.* 1996) as observed in this study.

A small number of the fifteen different bacterial sequence types characterised in cabbage aphid, particularly the types represented by only a single clone of 16S sequence, were probably environmental contaminants or transient bacteria in the aphid gut. The cabbage aphid sequence types represented by larger numbers of cloned sequences (Group 1 '*Pseudomonas'* types 8, 9 and 10 and Group 2 '*Erwinia'* types 12, 13, 14 and 15) are less

likely to be transient and therefore might influence aphid fitness. Often bacteria with similar capabilities and lifestyles group together and the phylogenetic relationships of bacteria can shed light on aspects of their ecology (Brown *et al.* 2000). Cabbage aphid sequence types found in the orders Enterobacteriales and the Pseudomonales were closely related to several pathogenic bacteria that cause plant diseases (*Erwinia* and *Pectobacterium* species) (Brown *et al.* 2000) and insect pathogens known to reduce aphid fitness (*P. aeruginosa* and *E. aphidicola*) (Harada and Ishikawa 1997; Grenier *et al.* 2006). The extent to which the bacterial types associated with the cabbage aphid influence aphid fitness depends on their titre in aphid tissues and degree of transmission between generations (Darby and Douglas 2003; Moran *et al.* 2008). Numerous observations have suggested that the secondary symbionts are also found outwith the mycetocytes in the aphid haemolymph, gut and reproductive organs (Fukatsu *et al.* 2000; Sandström *et al.* 2001; Darby *et al.* 2001; Sakurai *et al.* 2005). Although the likely location of the bacteria characterised in the cabbage aphid is the gut, they could also infect other tissues, including the reproductive tissues to establish a transovarial transmission route. The localisation of the different cabbage aphid bacteria could be established by a microscopic examination using fluorescent *in situ* hybridisation (FISH). FISH has been used successfully to localise symbiotic bacteria in aphids (Fukatsu *et al.* 1998; Fukatsu 2001; Fukatsu *et al* 2000; 2001; Darby *et al.* 2001; Sakurai *et al.* 2005) and other insects (Santo Domingo *et al.* 1998; Dunn and Stabb 2005). It should be possible to localise the cabbage aphid bacterium by designing fluorescent probes based on the 16S sequence for each bacteria, or group of bacteria, associated with the cabbage aphid. The Gram-staining technique used in this study was applied on homogenised aphid samples and therefore only yielded information regarding the morphology and physiology of the bacteria and not their location. Determining the location of bacteria associated with the cabbage aphid would provide information on their relationship with the host and their lifestyle and transmission capability between generations thus indicating the potential for affecting cabbage aphid fitness.

2.4.2 Resolution of phylogenetic relationships between bacteria could be improved by using alternative genes or tree drawing methods.

The candidate gene upon which phylogenetic analysis is based can greatly influence the accuracy of classification of different sequence types. The 16S gene was chosen for phylogenetic analysis in this study for three reasons. Firstly, 16S sequence could be generated from cabbage aphid samples by using universal primers which excluded *Buchnera* (Sandström *et al.* 2001). Universal PCR amplification of an alternative bacterial gene in aphid DNA samples would be considerably biased towards *Buchnera*. Secondly, the 16S gene exhibits sufficient variation to determine phylogenetic relationships between bacterial species within the same genus (Wertz *et al.* 2003). Thirdly, as the 16S gene is shared by all bacteria and is commonly used for phylogenetic classification of bacteria there is a wealth of bacterial 16S sequence in the NCBI database. Previous studies have successfully used the 16S gene for phylogenetic characterisation of the symbiont bacteria of insects (Fukatsu and Nikoh 1998; Fukatsu 2001; Fukatsu *et al.* 2000; Darby *et al.* 2001; Dunn and Stabb 2005; Sakurai *et al.* 2005) and therefore it was considered a suitable candidate gene for this study. However, the 16S gene encodes ribosomal proteins which have a key role in DNA translation, and thus is highly conserved across bacterial genera. Some studies have reported the inability of the 16S gene to group bacterial species accurately within different genera (Drancourt *et al.* 2000; Naum *et al.* 2008) indicating that while variation in 16S sequence can be used to infer taxonomic relationships between groups of bacteria (Naum *et al.* 2008) it is less effective at determining phylogenetic relationships between closely related taxa.

Despite the highly conserved nature of 16rRNA sequence many studies to classify taxonomic positions and phylogenetic relationships between bacterial taxa used this gene (Bennasar *et al.* 1996; Anzai *et al.* 2000; Bosshard *et al.* 2006; Brambilla *et al.* 2007). Several highly variable blocks, termed 'hypervariable regions' separated by regions of conserved sequence have recently been identified in 16S rDNA sequence (Naum *et al.* 2008) and used to separate bacterial species within the same genus (e.g. Hassan *et al.* 2007 *Enterobacter* species). However, to separate closely related species and improve phylogenetic resolution, specific sequences from different candidate genes can be used (Guasp *et al.* 2000; Hilario *et al.* 2004; Yu and Morrison 2004; Paradis *et al.* 2005; Nhung

et al. 2007). Non-coding regions, such as 16S–23S ITS, are under reduced selection pressure to conserve sequence compared to coding regions and show a greater percentage of mutations (Tyrrell *et al.* 1997). Thus, the 16S–23S ITS can be used to effectively group species within different genera (Guasp *et al.* 2000). Indeed the IGS region between the 16S and 23S ribosomal subunits generated by PCR in this study but not sequenced has also been used for classifying bacteria (Barry *et al.* 1991). Multiple housekeeping genes *atpD*, *carA*, *recA* and *rrs* have also been used to improve the resolution of phylogenetic analysis (Hilario *et al.* 2004; Yu and Morrison 2004). Often phylogenies generated by alternative genes are in agreement with the phylogenetic relationships estimated using 16S rRNA sequences but tend to give better discrimination between pairs of species (Guasp *et al.* 2000; Hilario *et al.* 2004; Paradis *et al.* 2005; Nhung *et al.* 2007).

Although phylogenetic resolution could have been improved in this study by choosing a candidate gene with greater variability, considerable additional effort would have been required to eliminate *Buchnera* sequence from the amplification process and to capture a representative sample of secondary bacterial diversity in cabbage aphid. The IGS region between the 16S and 23S subunits might be another suitable region for phylogenetic analysis as it can be amplified using the same 16S universal primers used for this study, therefore excluding *Buchnera*. The IGS is non-coding and consequently exhibits greater sequence variability than the 16S gene. However, good bootstrap separation between groups of cabbage aphid sequence types and short branch lengths indicating low within group variation indicated the 16S gene was sufficiently variable to differentiate the diverse bacterial sequence types associated with the cabbage aphid. In addition, the IGS region is smaller and infrequently sequenced; had this been the target region, the sequenced product for comparison would have been smaller (IGS = ~181 bp and 16S = 1500 bp) and there would be limited published sequence available for comparison. Thus, the 16S gene sequence was preferred due to availability of 16S database sequence for phylogenetic comparison.

The resolution of phylogenetic trees is also dependent on the algorithm that has been used to infer the degree of relatedness between the sequences. There are numerous tree drawing methods available to maximise the resolution of phylogenetic analysis, although maximum likelihood, the method used in this study, is the most established

method (Hobolth and Yushida 2005). Maximum likelihood describes evolution in terms of a discrete–state–continuous time Markov process on a phylogenetic tree (Hobolth and Yushida 2005). The neighbour joining method which has been popular for many years is a distance method for tree reconstruction based on pairwise comparison between sequences to cluster neighbouring sequences recursively (Hobolth and Yushida 2005). Bayesian methods such as Mr Bayes are a more recent addition to phylogenetic analysis that are now being widely used (Naum *et al.* 2008) and are particularly useful because they can incorporate secondary structure into the analysis. The maximum likelihood method was chosen for this study due to reduced vulnerability to gaps in the alignment compared to the neighbour joining method and the speed with which it can generate phylogenetic trees. Bayesian analysis takes considerably longer to run, but would improve phylogenetic resolution if secondary structure information was available and should be considered for future studies.

2.4.3 The survey of diverse bacterial types characterised was not exhaustive.

A key question about molecular studies to classify bacteria is whether the methods have captured a representative sample of the bacterial diversity. Although the diversity of bacteria captured in this study was sufficient for the purpose of the insect fitness studies, it cannot be considered an exhaustive characterisation. Firstly, phylogenetic analysis revealed that cabbage aphid type 11 was *Buchnera* which questioned the specificity and sensitivity of the universal bacterial 16S–23S primers that should have excluded *Buchnera* sequence. The specificity of universal bacterial primers for molecular profiling of bacteria has been questioned in recent research as some can amplify eukaryotic and archaeal DNA (Huws *et al.* 2007). Given the large range of primers and PCR conditions used to amplify bacterial DNA, conditions with the potential to bias results by amplifying non-target sequences is relatively high (Huws *et al.* 2007). In this study, sequence data indicated that the universal primers (10F and 480R Sandström *et al.* 2001) were bacteria specific. As *Buchnera* sequence was only represented by a single clone out of several hundred, and as no other cabbage aphid sequence type grouped closely with the *Buchnera* sequence, non-specific amplification of *Buchnera* did not appear to influence the results.

Secondly, rarefaction analysis suggested that the sequence types with multiple representative clones had been exhaustively sampled but that the diversity of the sequence types only represented by a single clone had not been adequately quantified. The drawback of using rarefaction analysis to determine the point at which saturation of sampling had occurred is that it is only efficient at estimating sampling intensity when sequence types are neither extremely rare nor extremely common, which could occur as a consequence of PCR bias (Gotelli and Colwell 2001). Some studies have reported PCR bias towards amplification of common bacterial types (Becker *et al.* 2000). In complex samples amplicons generated from different bacterial types compete leading to suppression of the less abundant bacterial type by amplification of the dominant bacterial type (Becker *et al.* 2000). Rarefaction assumes that the number of occurrences of a sequence type reflects the sampling intensity (Gotelli and Colwell 2001), but if one sequence type is amplified preferentially, the number of occurences of that sequence type will reflect the frequency of the cloned sequence and not the sampling intensity. The same would be true of uncommon sequence types that are amplified rarely leading to an underestimation of the abundance of rare types. Alternative 16S–23S primer combinations revealed there was no evidence to suspect PCR bias was occuring using the 10F/480R primers and therefore that sequence types were represented by a large or small number of clones because they were either common or rare in the DNA samples. More exhaustive sampling of the clone library might have lead to the detection of additional rare sequence types. However, for the purpose of studying effects on aphid fitness the number of common bacterial types identified (>4) was considered sufficient and indicative of cabbage aphid bacterial community.

Finally, bias in the DNA extraction method towards Gram-negative bacteria could mean that a proportion of the bacterial diversity in cabbage aphid was not captured in this study. Other studies have reported the presence of Gram-positive bacteria in aphids (Grenier *et al.* 1994; Nakabachi *et al.* 2003). DNA extraction from Gram-positive bacteria requires enzyme (lysozyme) digestion of the peptidoglycan layer, which was not employed in this study. However, Gram-positive bacteria were not detected by Gram staining of homogenised cabbage aphids from different clonal lines, indicating that the contribution of Gram-positive bacteria to bacterial diversity in cabbage aphid was small.

2.4.4 Conclusions

Microscopy revealed that there were two distinct morphological types of bacteria associated with the cabbage aphid. The large round cocci most likely represented *Buchnera* while the rod-shaped bacteria could belong to a wide diversity of secondary facultative or free-living bacteria. The diversity and phylogenetic position of the secondary bacteria suggest that they belong to free living groups that could include plant and insect pathogens and therefore influence the fitness of cabbage aphids. PCR indicated that there was variation in the presence and absence of secondary bacteria across cabbage aphid lines that could provide a basis for examining the effect of natural bacterial infections on aphid fitness and to develop high throughput molecular quantification methods. The most common cloned sequence types of bacteria characterised in cabbage aphid could be split into two main groups comprising two bacterial orders: Group 1 '*Pseudomonas'* types 8, 9 and 10 and Group 2 '*Erwinia'* types 12, 13, 14 and 15. These two groups will form the basis for designing a real-time quantitative PCR assay to screen further cabbage aphid clonal lines for their bacterial complement.

3. Molecular quantification of the bacteria associated with the cabbage aphid.

3.1 Introduction

3.1.1 High-throughput methods for determining bacterial complement in aphids

Molecular characterisation of the bacteria associated with cabbage aphid (*Brevicoryne brassicae*) revealed at least fifteen different bacterial sequence types, of which the majority grouped phylogenetically within the orders Enterobacteriales and Pseudomonales. Both of these orders include known insect pathogens. Consequently, methods for quantification of the two groups named *Pseudomonas* (Group 1 Pseudomonales) and *Erwinia* (Group 2 Enterobacteriales) were investigated to determine the infection status of the cultured cabbage aphid lines. Sequencing data (Chapter 2) suggested that the relative quantity of bacterial types would vary across the cabbage aphid lines. The variation in bacterial complement could relate to numerous factors including the host plant from which the aphids were originally collected or genotypic variation in susceptibility to infection. Various molecular methods that target specific gene sequences have been employed to establish the bacteria present in aphid lines, including cloning and sequencing (Nakabachi *et al.* 2003), quantitative real-time PCR (qPCR) (Sakurai *et al.* 2005; Chandler *et al.* 2008), Southern Blotting (Unterman *et al.* 1989), restriction digests combined with denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Haynes *et al.* 2003). The 16S sequence and clone library generated for the initial characterisation of the cabbage aphid sequence types (Chapter 2) provided a basis for the development and testing of two different high through-put approaches, T-RFLP and qPCR.

3.1.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) was initially developed to assess the general diversity of bacterial communities and shifts in patterns of diversity (Liu *et al.* 1997; Hongoh *et al.* 2005) but can also be applied in a directed manner

for identification of single species from samples of DNA (e.g. Burke *et al.* 2005). The chosen DNA template in a complex sample is amplified by PCR using universal primers and the PCR product is digested with restriction enzymes that have a 4–6 bp recognition site (Liu *et al.* 1997). The universal forward primer is fluorescently labelled at the 5' end facilitating the T-RF size analysis by automated DNA sequencing using fluorescent detection technology (Liu *et al.* 1997). In directed T-RFLP, the restriction enzymes cut each bacterial 16S sequence type at particular nucleotide positions to generate terminal restriction fragments (T-RFs) of a specific size for each bacterial type (Burke *et al.* 2005). Restriction-digestion of fluorescently end-labelled PCR product yields one labelled terminal fragment and one or more unlabelled fragments (Liu *et al.* 1997). The digested DNA fragments are separated by capillary electrophoresis on the automated DNA sequencer which only detects the fluorescently labelled ends (Liu *et al.* 1997). Electropherograms are generated in which the intensity of the fluorescent signal is plotted against fragment size, with a labelled fragment length marker for reference. Each peak in the T-RFLP profile corresponds to a different 16S sequence variant (T-RF) and the pattern of peaks can be used to indicate the diversity of bacterial types in complex samples (Liu *et al.* 1997). A carefully designed directed T-RFLP approach using known bacterial sequence can generate signature peaks assigned to specific bacterial groups, e.g. each different cabbage aphid sequence type. Directed T-RFLP has been applied successfully to assess bacterial diversity in pea aphid (*A. pisum*) populations (Haynes *et al.* 2003; Ferrari *et al.* 2004). Consequently, a directed T-RFLP based on 16S sequence was designed to assess the bacterial complement of cabbage aphid lines.

3.1.3 Real-time quantitative polymerase chain reaction.

Real-time quantitative PCR (qPCR) is an established alternative method to quantify the primary symbiont *Buchnera* (Komaki and Ishikawa 2000; Koga *et al.* 2003; Nakabachi *et al.* 2003; Plague *et al.* 2003; Sakurai *et al.* 2005; Douglas *et al.* 2006b; Oliver *et al.* 2006; Dunbar *et al.* 2007; Wilkinson *et al.* 2007; Chandler *et al.* 2008) and the secondary symbionts *H. defensa* (Oliver *et al.* 2006; Chandler *et al.* 2008), *R. insecticola* (Chandler *et al.* 2008) and *S. symbiotica* (Koga *et al.* 2003; Oliver *et al.* 2006; Wilkinson *et al.* 2007) in aphids. Optimised real-time PCR assays based on efficient fluorescent detection

chemistries can be used to determine the number of DNA copies of a specific sequence in a complex sample i.e. the number of copies of a bacterial gene in a sample of DNA extracted from aphids. This approach was adapted to quantify the two groups of bacteria characterised in the cabbage aphid based on the bacterial 16S gene.

Polymerase chain reaction (PCR) can be used to amplify a large number of identical copies of any nucleic acid 'target' sequence present in a sample of DNA through a series of denaturation, annealing and elongation cycles (Mullis and Fobona 1987). Oligonucleotide primers are designed to complement each end of the target DNA sequence and are extended towards each other by a DNA polymerase thermostable enzyme such as *Taq* Polymerase (Mullis and Fobona 1987). Initially, a high temperature is applied to denature the double strands of DNA, then the temperature is lowered to allow the primers to anneal to the template, and finally the temperature is increased to the optimum for *Taq* polymerase which extends the primers towards each other by incorporating dNTPs (deoxyribonucleotide triphosphates), using the target sequence as a template (Mullis and Fobona 1987). The number of target DNA copies should double during each cycle of PCR until one of the reaction reagents becomes limiting and the reaction reaches a plateau (Mullis and Fobona 1987). In 'end-point' PCR, the product is typically separated by gel-electrophoresis, to determine product size, and then used for down-stream reactions such as cloning and sequencing for molecular characterisation (Chapter 2). However, as the amount of product generated by 'end-point' PCR the reaction does not always accurately reflect the initial template copy number (Kubista *et al.* 2006), real-time qPCR was developed as an alternative quantitative method. In real-time qPCR, fluorescent molecules are monitored using an optical thermocycler (e.g. H7500 FAST block ABI system). The thermocycler provides excitation of fluorescent molecules that are either free in solution (SYBR® Green I) and fluoresce when bound to the target DNA molecules or that are covalently bound to an oligonucleotide probe (Taqman®); fluorescent emission is quantified over the course of the PCR reaction to determine the number of DNA copies in 'real-time' (Pryor and Wittwer 2006). Taqman® probes are often used in real-time qPCR assays and differ from other methods such as SYBR® Green I because the fluorescent molecules are covalently linked to an oligonucleotide probe specific to the target DNA sequence. As Taqman® probes allow detection of a specific target within a complex sample and are sensitive enough to

detect single nucleotide polymorphisms, they were chosen for this study to discriminate between highly conserved 16S sequences of cabbage aphid secondary bacteria (Chapter 2).

3.1.4 Taqman® detection chemistries for real-time qPCR.

Taqman® probes incorporate a fluorophore e.g. FAM (6-carboxyfluorescein) attached to the 5' end and a quencher e.g. BHQ (Black Hole Quencher®) attached to the 3' end. A fluorophore is a molecule that first absorbs light of a specific wavelength then emits light of a certain longer wavelength, while a quencher dissipates energy in the form of light or heat that it has accepted from the fluorophore. When Taqman® probes are used the fluorophore (FAM) is excited by the optical thermocycler and passes its energy via FRET (Fluorescence Resonance Energy Transfer) to the quencher (BHQ) that quenches the emission spectra by releasing the energy as heat (Heid *et al*. 1996). On each annealing step of the real-time PCR reaction, the probe binds to the target amplicon, and as the Taq polymerase extends from the primer it displaces the 5' end of the probe (Heid *et al*. 1996). The 5'–3' exonuclease activity of the Taq polymerase enzyme causes the probe to degrade separating the fluorophore and the quencher in solution which releases the quenching of the fluorescent emission of the fluorophore (FAM) resulting in a detectable increase in fluorescent emission of the appropriate wavelength for the fluorophore (FAM = 518nm) above the background threshold (Heid *et al*. 1996). Quantification of the amount of DNA copies of a target amplicon is based on the number of PCR cycles completed before the background fluorescence is exceeded (Heid *et al*. 1996). The real-time PCR reaction can be split into three phases (Fig. 3.1) starting with amplification under background fluorescence, followed by exponential amplification above the background fluorescence once the background threshold has been exceeded, and then a plateau in amplification occurs as the reaction becomes limited by substrate availability (Pfaffl 2004). The optical thermocycler monitors the real-time PCR reaction and records the number of amplification cycles required to obtain a particular number of DNA copies of a target sequence by producing an amplification plot of fluorescence level against cycle number (Fig. 3.1) (Kubista *et al.* 2006). The cycle number (Ct value) at which the fluorescent signal is detected above a defined background threshold during the exponential phase of amplification of the target sequence is recorded and used to quantify the number of copies of the target sequence in

the reaction (Kubista *et al.* 2006). The amount of amplified target DNA is directly proportional to the amount of target initially present in the reaction only during the phase when the level of florescence is increasing exponentially (Pfaffl 2004). Increasing the number of copies of target DNA sequence leads to more rapid signal detection, generating smaller Ct values (Kubista *et al.* 2006). Differences between samples in the number of target DNA copies can be calculated based on the assumption that the number of DNA copies doubles at each cycle in the exponential phase of amplification (Kubista *et al.* 2006).

To interpret differences in Ct values they must be correlated with other quantitative values (Kubista *et al.* 2006). The quantitative values used to make the Ct values meaningful depend on the method of quantification. In 'relative' quantification, the number of copies of the target gene (bacterial 16S) is quantified relative to an endogenous reference gene, which is present in all samples. The aphid nuclear gene for elongation factor 1-α (*Ef1-α*) was chosen as a suitable reference gene for this study to indicate the quantity of aphid tissue in the sample. Elongation factor genes are highly conserved 'house-keeping' genes that have multiple roles in eukaryotes (Tatsuka *et al.* 1992). *Ef1-α* is a GTP binding protein that catalyses the binding of aminoacyl-transfer RNAs to the ribosome forming an essential component of the eukaryotic translational apparatus (Tatsuka *et al.* 1992). There are a number of methods used for relative quantification that vary in their complexity. The most widely used and simplest method is the comparative cycle threshold method which is also referred to as the 'delta delta Ct' (ΔΔCt) method. The ΔΔCt method allows direct comparison between the Ct values generated by the target and reference genes in an unknown sample relative to a calibrator sample (Livak and Schmittgen 2001). To use the ΔΔCt method, the amplification efficiency of the primers for the target and reference genes must be close to 100% and approximately equal, which can be tested by comparison of standard curves. The amount of target gene in an unknown sample is calculated relative to the amount of template in another 'calibrator' sample, which contains both target and reference sequences with a constant ratio (Livak and Schmittgen 2001). The calibrator sample could be an untreated sample or a sample taken prior to the start of an experiment. A study investigating the relative quantity of secondary symbiont bacteria across different aphid lines could use an aposymbiotic aphid line as a calibrator. Frequently, however, the sample with the lowest expression of the target gene is used as a calibrator. The difference

between the Ct values generated for the target and reference genes for each sample (ΔCt value) is normalised against the difference between the Ct values generated for the target and reference genes for the calibrator sample (ΔΔCt value) to calculate the quantity of the target relative to the calibrator ($RQ = 2^{-\Delta\Delta Ct}$) (Section 3.2.3.5; Appendix 1) (Livak and Schmittgen 2001). The ΔΔCt method can therefore be used to compare the relative quantity of Group 1 and 2 bacteria, using the 16S gene as a target, across each cabbage aphid line. Results from the screening process using the real-time qPCR assays would form the basis for the selection of cabbage aphid lines with varying bacterial infections to be used in experiments to investigate the effect of the two bacterial groups on cabbage aphid fitness.

Two high-throughput methods, T-RFLP and real-time qPCR, were therefore available to determine the bacterial complement of the cabbage aphid. The aim of this study was to determine which method was optimal for accurate detection of the bacteria associated with the cabbage aphid for subsequent screening of the cultured cabbage aphid lines.

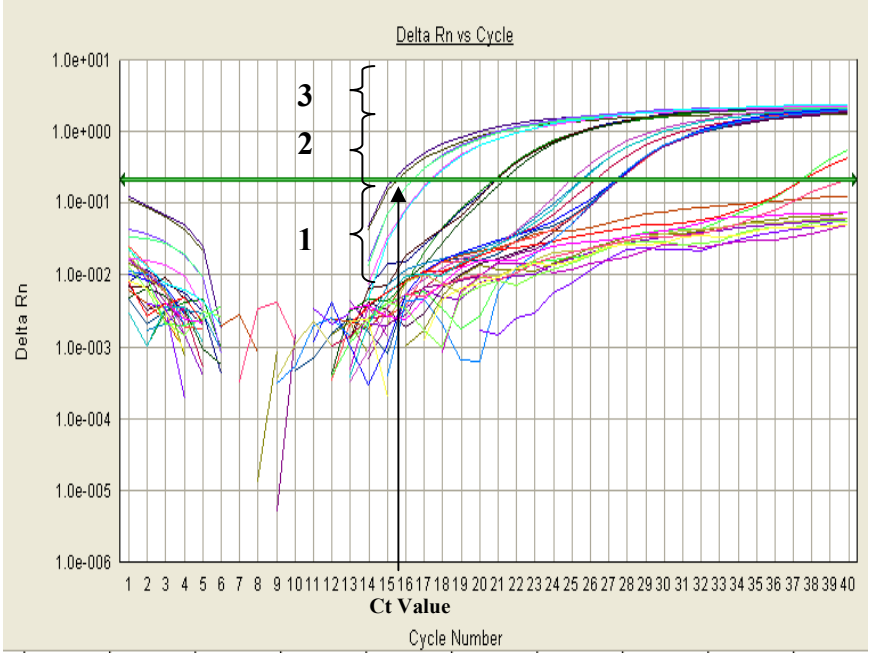


Figure 3.1: The real-time PCR reaction can be split into three phases; 1) amplification under background fluorescence, 2) exponential amplification above the background fluorescence, 3) plateau in amplification as the reaction becomes substrate-limited.

3.2 Materials and Methods

3.2.1 Experimental Material

The cabbage aphid lines reared in culture conditions (Section 2.2.1) screened using the Taqman® real-time PCR assay are shown in Table 3.1. DNA was extracted from samples of multiple individuals (~50 aphids) of approximately equal tissue mass from each aphid line using a DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, California) as described in Chapter 2 (Section 2.2.3).

Table 3.1: Details of cabbage aphid **(B. brassicae)** *cultures collected and used as experimental material.*

Code	Location	Brassica Variety	Cultivated or Feral
ELC0604	Tayport, Fife	Cabbage	Cultivated
ELC0605	Dundee, Tayside	Brussels Sprout	Cultivated
ELC0606	Letham, Fife	Broccoli	Cultivated
ELC0607	Tayport, Fife	Purple Sprouting Broccoli	Cultivated
ELC0610	Balmullo, Fife	Kale	Feral
ELC0611	Dundee, Tayside	Cabbage	Cultivated
ELC0612	Dundee, Tayside	Brussels Sprout	Cultivated
ELC0613	Tayport, Fife	Brussels Sprout	Cultivated
ELC0618	Dundee, Tayside	Cauliflower	Cultivated
ELC0619	Invergowrie, Perthshire	Turnip	Cultivated
ELC0701	Invergowrie, Perthshire	Oilseed Rape	Cultivated
ELC0703	Tayport, Fife	Brussels Sprout	Cultivated
ELC0801	Snainton, Yorkshire	Brussels Sprout	Cultivated
ELC0803	Snainton, Yorkshire	Cabbage	Cultivated
ELC0604P	Tayport, Fife	Cabbage	Cultivated
ELC0606P	Letham, Fife	Broccoli	Cultivated

3.2.2 Directed T-RFLP to determine bacterial complement in cabbage aphid.

Selection of suitable restriction enzymes for each cabbage aphid sequence type was based on *in silico* analysis of 5'-terminal restriction fragments (T-RFs) from 16S cabbage aphid bacterial sequence using the T-RFLP analysis program TRiFLe (Junier *et al.* 2008). TRiFLe generates theoretical T-RFs from sets of sequence by simulating PCR amplification and digestion with restriction enzymes (Junier *et al.* 2008). The output from TRiFLe provided a means for selecting the optimal enzymes to detect each different cabbage aphid sequence type based on the predicted T-RF sizes on a T-RFLP profile. The restriction enzymes that provided unique T-RFs for the greatest numbers of cabbage aphid sequence types were chosen for use in the T-RFLP assay. Due to the highly conserved nature of the 16S sequence it was not possible to separate out each individual sequence type and therefore some closely related types were often represented by a single T-RF e.g. types 6 and 7 and types 8 and 9. To resolve this problem samples were digested in parallel with four different restriction enzymes resulting in multiple T-RFLP profiles each of which resolved some sequence types but not others. After comprehensive analysis of all restriction digest options it was determined that two double digests (i.e. parallel digests including two restriction enzymes rather than a single enzyme) were necessary to generate specific T-RFs for each of the fifteen cabbage aphid sequence types. The restriction enzymes *Sfa*NI (5'-GCATC$(N)_2$-3', 3'-CGTAG$(N)_9$-5') and *Bbs*I (5'-GAAGAC$(N)_5$-3', 3'-CTTCTG$(N)_6$-5') were used for Digest 1 while the restriction enzymes *Msp*A1I (5'-CMGCKG-3', 3'-CMGCKG-5') and *Tsp*509I (5'-AATT-3', 3'-TTAA-5') were used for Digest 2. The recognition sites for each enzyme were verified using the multiple alignment editor Genedoc (http://www.psc.edu/biomed/genedoc).

Once suitable enzymes had been chosen 16S rDNA gene fragments were amplified from total cabbage aphid DNA using the universal bacterial primers 10F 5′-AGTTTGATCATGGCTCAGATTG-3′ (Sandström *et al.* 2001) and 1056R 5'-ACACGAGCTGACGACAGCCA-3' (Allen *et al.* 2001). The 10F forward primer was labelled at the 5' end with the phosphoramidite dye 6-FAM (EUROGENTEC Ltd, Belgium). PCR amplification conditions were the same as in the previous chapter (Section 2.2.4.2). Amplification consisted of 30 cycles of 94ºC for 1 minute to denature the DNA,

55ºC for 1 minute for annealing of the primers and 72ºC for 2 minutes elongation by the *Taq* polymerase, with the denaturing step extended to 5 minutes in the first cycle and the elongation step increased to 8 minutes in the final cycle. The PCR reaction mixture was as described in Chapter 2 (Section 2.2.4.2). A sample of PCR product (~3 µl) from each amplification was run on a 1% agarose gel to verify that the PCR reaction was successful (Section 2.2.4.1). To purify the remaining sample (~22 µl) 2 µl sodium acetate, 1 µl of glycogen and 32 µl of 100% ethanol were added to each well and the plate was vortexed to mix. Following 1.5 hours incubation in a -80ºC freezer the plate was centrifuged at 1600 *g* (Sigma 4K15 Centrifuge, Sigma Laboratories Centrifuges) and 4ºC for 15 minutes. Glycogen addition allowed the DNA pellet to be visualised so that the supernatant could be removed by pipetting. The remaining DNA pellets were washed with 100 µl of 70% ethanol (cooled to -20ºC). The samples were centrifuged for 5 minutes at 1600 *g*, the supernatant removed and the samples were air dried for 25 minutes at ~21ºC. The washed DNA pellet was resuspended in 10 µl of MBG water and the final concentration of DNA was measured using a Full Spectrum UV/Vis NANODrop Spectrophotometer (ND-1000, Labtech International).

Purified samples were digested either with 1 Unit of *Sfa*NI (1 Unit is defined as the amount of enzyme required to digest 1 µg of λ DNA in 1 hour at 37ºC in a total reaction volume of 50 µl) and 2.5 Units of *Bbs*I (37ºC for 2 hours) or sequentially with 5 Units of *Msp*A1I (37ºC for 2 hours) followed by 5 Units of *Tsp*509I (65ºC for 2 hours). Each reaction included 1X NE buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol) and the digest with *Msp*A1I was supplemented with Bovine Serum Albumin (BSA) at 1 mg/ml. All digests were NE Biolabs specified restriction endonuclease digests and reagents and enzymes were all synthesised by New England Biolabs (UK) Ltd, Hertfordshire, United Kingdom. Dilutions (1:10) of each sample were prepared and 1 µl of each dilution was aliquoted into a 96 well low profile PCR plate (ABgene). A mastermix of 895 µl of Hi-Di™ Formamide (Applied Biosystems, Warrington, UK) which facilitates electrokinetic injection on the capillary electrophoresis system and 5 µl of the size standard, MapMarker® 1000 carboxy-X-rhodamine (ROX) labelled (Bioventures, Cambridge, U.K) was prepared and 9 µl aliquots were added to each sample in the 96 well plate. The samples were mixed by pipetting and run on an ABI3730 capillary sequencer (Applied Biosystems,

Warrington, UK) to separate the terminal restriction fragments. GeneMapper version 3.7 (Applied Biosystems, Warrington, UK) was used to detect the presence or absence of fluorescence peaks at the expected T-RF sizes and any additional peaks. Ideally, the observed T-RF peaks would match the expected peaks (+/- 5 bp) and there would be few additional peaks in the T-RFLP profiles.

3.2.3 Real-time (Taqman®) quantitative PCR assay development for quantification of two bacterial groups.

3.2.3.1 Real-time Taqman® assay design.

The real-time PCR assay incorporated Taqman® chemistry using a sequence specific probe hybridising between specific forward and reverse primers for the two groups of bacteria characterised in cabbage aphid (Chapter 2). Two sets of real-time PCR primers were designed to amplify a short amplicon (~80 bp) of the target nucleotide sequence from the 16S gene in the two groups of bacteria (Table 3.2). Potential primer binding sites were identified using Primer Express® software (Applied Biosystems, Warrington, UK) and alignments were viewed in Genedoc and TOPALi version 2 (Appendix 2). Primers and probes were checked for efficiency using Netprimer software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html), which calculates melting temperatures and GC richness and assigns an efficiency score to each primer. To confirm that the primers and probes were specific for each of the two target types of bacteria and did not cross-react the sequences were compared with database sequence using a Basic Local Alignment Search Tool (BLAST) search on the NCBI sequence database. Using the same approach, a specific probe was also designed for each group of bacteria between the forward and reverse primer sites on the 16S gene (Appendix 2; Table 3.2). Probes for the two bacterial groups were labelled at the 5' end with the reporter dye FAM (6 – carboxyfluorescein) and at the 3' end with the universal quencher dye BHQ-1 (Black Hole Quencher®). In each case the reverse complement of the probe was synthesised to maximise the number of cytosine (C) nucleotides as recommended by the Applied Biosystems criteria for optimal Taqman® probe design (Real-time PCR Systems Chemistry Guide, Applied Biosystems). The level of 16S sequence conservation between the cabbage

aphid bacterial sequence types was very high (Appendix 2), and thus the specificity of the probes for Group 1 and Group 2 bacteria was based on only a few bases difference from the other sequence types (Appendix 2). Taqman® probes should be capable of detecting single nucleotide polymorphisms (Real-time PCR Systems Chemistry Guide, Applied Biosystems) and therefore, the assay should be able to differentiate Group 1 and Group 2 bacteria from the other bacterial types.

Primers and probes were designed for the reference gene aphid elongation factor 1 α (*Ef*1-α) for relative quantification. *Ef1*-α was quantified using the forward primer ApisEF1-422F (Sakurai *et al.* 2005) and a reverse primer ApisEF1-537R that was designed as part of this study (Table 3.2). Direct sequencing of the *Ef1*-α product generated with published *Ef1*-α primers (Sakurai *et al.* 2005) designed for pea aphid revealed nucleotide polymorphisms in the cabbage aphid sequence compared with that from pea aphids (Appendix 2). The probe designed for cabbage aphid *Ef1*-α was 5'-labelled with another reporter dye Yakima Yellow® and 3'-labelled with the universal quencher dye BHQ-1 (Table 3.2) to facilitate duplexing of two different fluorophores in a single reaction.

Table 3.2: Real-time (Taqman®) primer and probe sequences.

Primers	**Name**	**Sequence (5'-3')**	**Gene**	**Assay**
Forward	CABAC2-695F	GTAGCGGTGAAATGCGTAGATATA	16S	Group 1 bacteria
Reverse	CABAC2-762R	CGCTTTCGCACCTCAGTGT	16S	Group 1 bacteria
Forward	CABAC1-155F	AAACGGTAGCTAATACCGCATAAC	16S	Group 2 bacteria
Reverse	CABAC1-269R	CTAGGGATCGTCGCCTAGGT	16S	Group 2 bacteria
Forward	ApisEF1-422F	CTCTGGATGGAATGGAGACAACA	*Ef1-α*	Aphid *Ef1-α*
Reverse	ApisEF1-537R	GACCGTCGGCCTTTCCTT	*Ef1-α*	Aphid *Ef1-α*
Probes				
Probe	CABAC2-718P (FAM- BHQ-1)	CCTTCGCCACTGGTGTTCCTTCC	16S	Group 1 bacteria
Probe	CABAC1-228P (FAM- BHQ-1)	CCTACTAGCTAATCCCATCTGGGCA CAT	16S	Group 2 bacteria
Probe	ApisEF1-506P (Yakima yellow-BHQ-1)	CTTTACGTTCAACATTCCATCCTTG AACC	*Ef1-α*	Aphid *Ef1-α*

3.2.3.2 General real-time PCR conditions.

Real-time PCR (SYBR®) reactions were performed using an ABI 7500 FAST Real-time PCR System (Applied Biosystems, Warrington, UK). Samples (1 µl) of DNA template (diluted 1/25 for initial concentrations <100 ng/µl and 1/50 for concentrations >100ng/µl) were added to 11 µl of reaction mix in a 96-well qPCR subskirted plate (Eurogentec, Belgium). A FAST BLUE qPCR MasterMix Plus Low ROX kit (Eurogentec, Belgium) was used for all qPCR Taqman® reactions. The PCR reaction mixture contained 1x PCR reaction buffer (dNTPs, Meteor*Taq* DNA polymerase, 4 mM $MgCl_2$, blue dye, ROX passive reference and stabilisers), 100 nM probe and 1 µl DNA extract in a 25 µl reaction. The reaction conditions were 95ºC for 5 minutes then 45 cycles of 95ºC for 3 seconds, 60ºC for 30 seconds and 72ºC for 10 seconds. Primer concentration matrices (150, 300 and 900 nM 3 x 3 set up; User Bulletin 2, Applied Biosystems) were tested to optimise primer concentrations and reaction efficiency was determined across a four point dilution series (1:1 to 1:100) at each matrix concentration position. The optimal primer concentration combination was one that gave the lowest threshold cycle (Ct) value, and this was 300 nM for both primers. The Ct values for each PCR reaction were calculated automatically by the ABI 7500 FAST Real-time PCR Systems software. For each primer and probe combination, replicate four control reactions containing 1 µl of water instead of DNA were included.

3.2.3.3 Optimisation of singleplex real-time PCR assays

The amplification efficiency of the target gene (bacterial 16S or aphid *Ef1-α*) was determined for three replicate dilutions (1/1 to 1/10,000) of total DNA extracted from appropriate cabbage aphids lines. A standard curve plot of Ct values against log[dilution of DNA template] (Fig. 3.2) allowed the efficiency of each assay to be assessed against the optimal criteria (slope >3.1, R^2 >0.985 User Bulletin 2, Applied Biosystems). The standard curves indicated that assays for the Group 2 bacteria (CABAC1) and *Ef1- α* fit these criteria but that the assay for Group 1 bacteria (CABAC2) was slightly less efficient (Fig. 3.2).

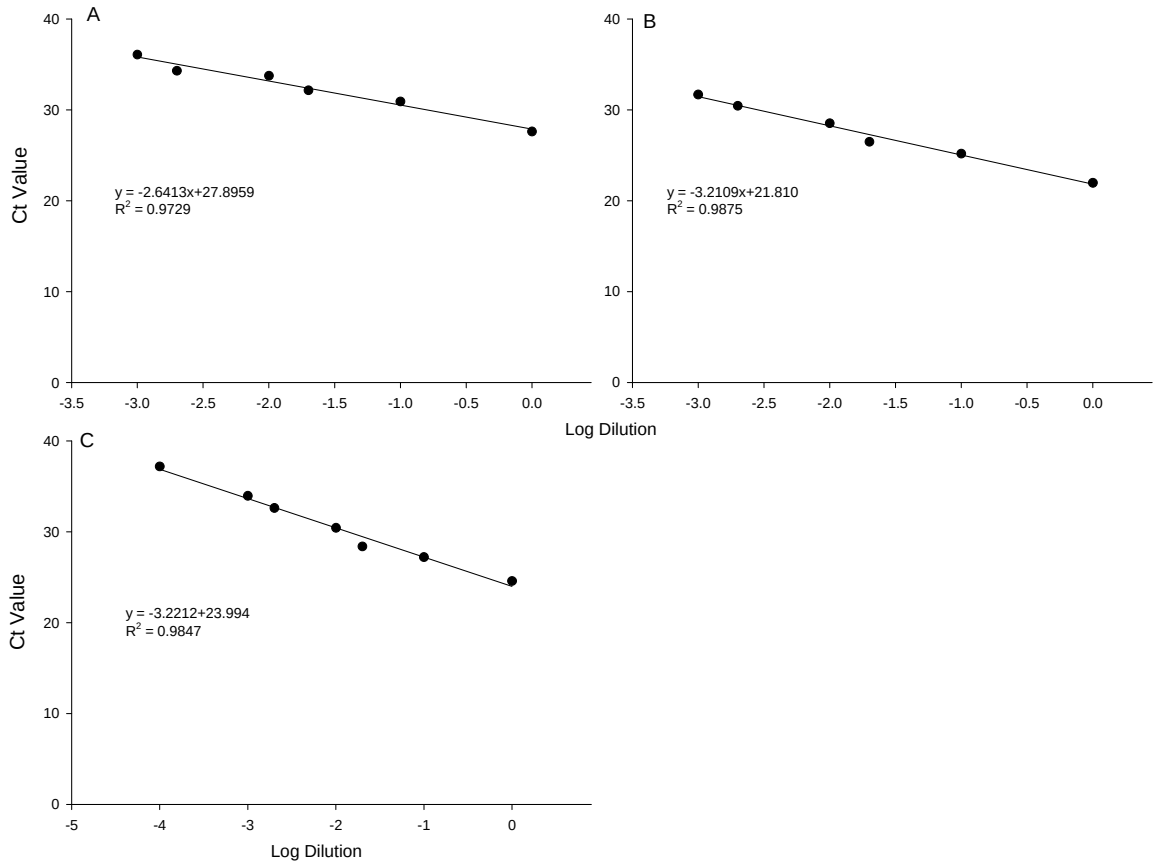


Figure 3.2: Standard curves for detection of (A) Group 1 bacteria using probe CABAC2, (B) Group 2 bacteria using probe CABAC1 and (C) the aphid* Ef1-α *reference gene using probe ApisEF1.

3.2.3.4 Optimisation of duplex real-time PCR assays.

A duplex PCR assay amplifies two separate DNA targets (e.g. bacterial 16S and aphid *Ef1-α*) in the same reaction. Two sets of primers one for each target and the accompanying probes labelled with different fluorescent molecules (e.g. FAM and Yakima Yellow) are present in each reaction (Table 3.2). Tandem reactions with two sets of primers might however compete and thus inhibit amplification efficiency. Amplification efficiency for the target and reference genes was determined as described above in single reactions for each primer pair and in duplex reactions for each combination of primer pairs. Comparison of the singleplex assays (Fig. 3.2) and duplex assays (Fig. 3.3) indicated no obvious reduction in the detection threshold for either gene in the duplex assays. Note that for the CABAC2 probe, the slope was less than the minimum value 3.1 both in the singleplex and duplex assays (Fig. 3.2A and 3.3B).

Using duplex assays reduced the number of reactions by half, thus decreasing the costs and increasing the speed of sample processing to determine the relative amounts of Group 1 and Group 2 bacteria in the different cabbage aphid lines.

3.2.3.5 Relative quantification using the comparative Ct method.

The comparative cycle threshold (Ct) method of relative quantification (ΔΔCt method) quantifies the number of copies of the target gene relative to a reference sample (calibrator) using arithmetic formulas. Ct values obtained for the target gene (16S) and endogenous control (*Ef1-α*) in the calibrator and test samples were used to calculate two parameters, $\Delta Ct = Ct_{target} - Ct_{endogenous\ control}$ and $\Delta\Delta Ct = \Delta Ct_{test\ sample} - \Delta Ct_{calibrator}$. The relative amount of target gene (16S) in each sample was calculated using the formula $2^{-\Delta\Delta Ct}$ i.e. the target normalised to the endogenous control and relative to the calibrator (see Appendix 1 for derivation of the formula) (Applied Biosystems, User Bulletin 2).

To use the ΔΔCt method, the amplification efficiencies of the reference and target genes must be approximately equal, indicated by a slope of <0.1 of ΔCt plot against log [DNA dilution] (User Bulletin 2, Applied Biosystems). Amplification efficiency was determined as described above. For both duplex assays, a plot of ΔCt against log [DNA dilution] gave a regression line slope <0.1 indicating equal amplification efficiencies for the target and endogenous genes (Fig. 3.4).

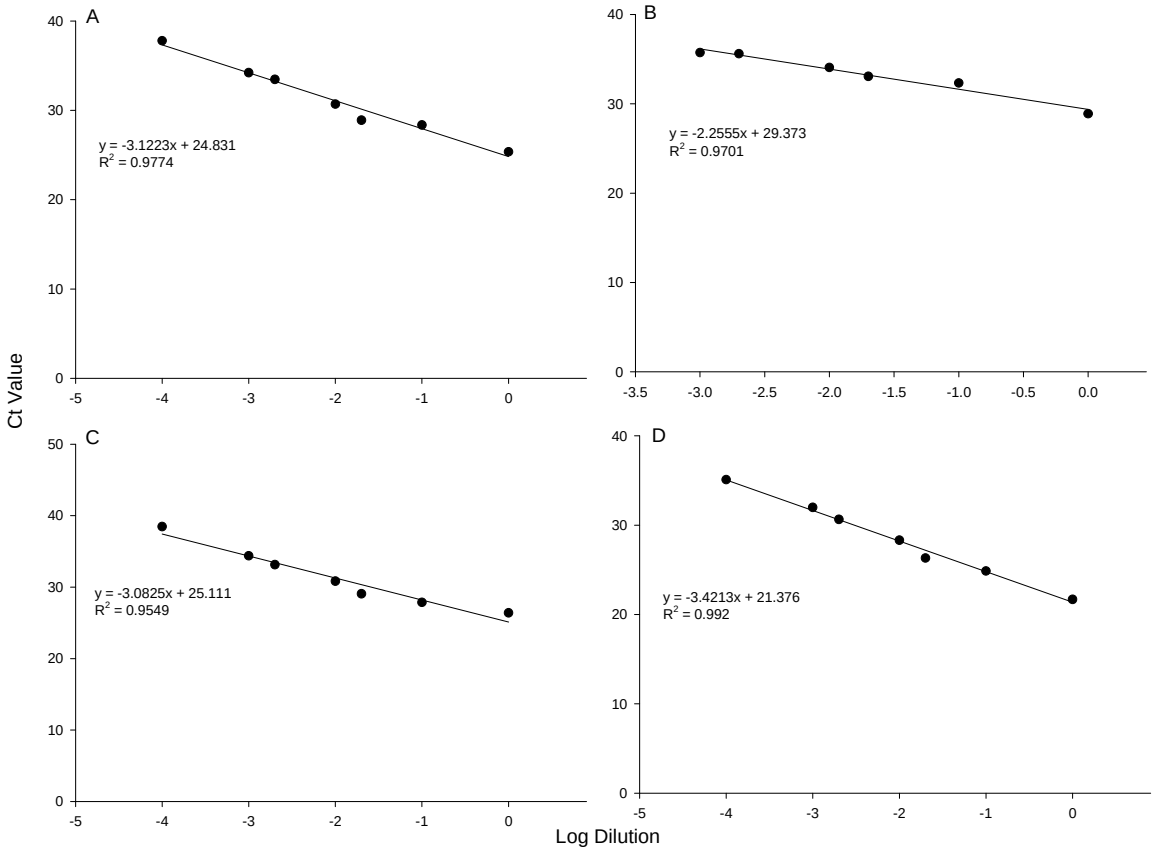


Figure 3.3: Standard curves for duplex assays to detect (A and B) Group 1 bacteria using probe CABAC2 with probe ApisEF1 and (C and D) Group 2 bacteria using probe CABAC1 with probe ApisEF1. (A = ApisEF1 efficiency with CABAC2, B = CABAC2 efficiency with ApisEF1, C = ApisEF1 efficiency with CABAC1 and D = CABAC1 efficiency with ApisEF1)

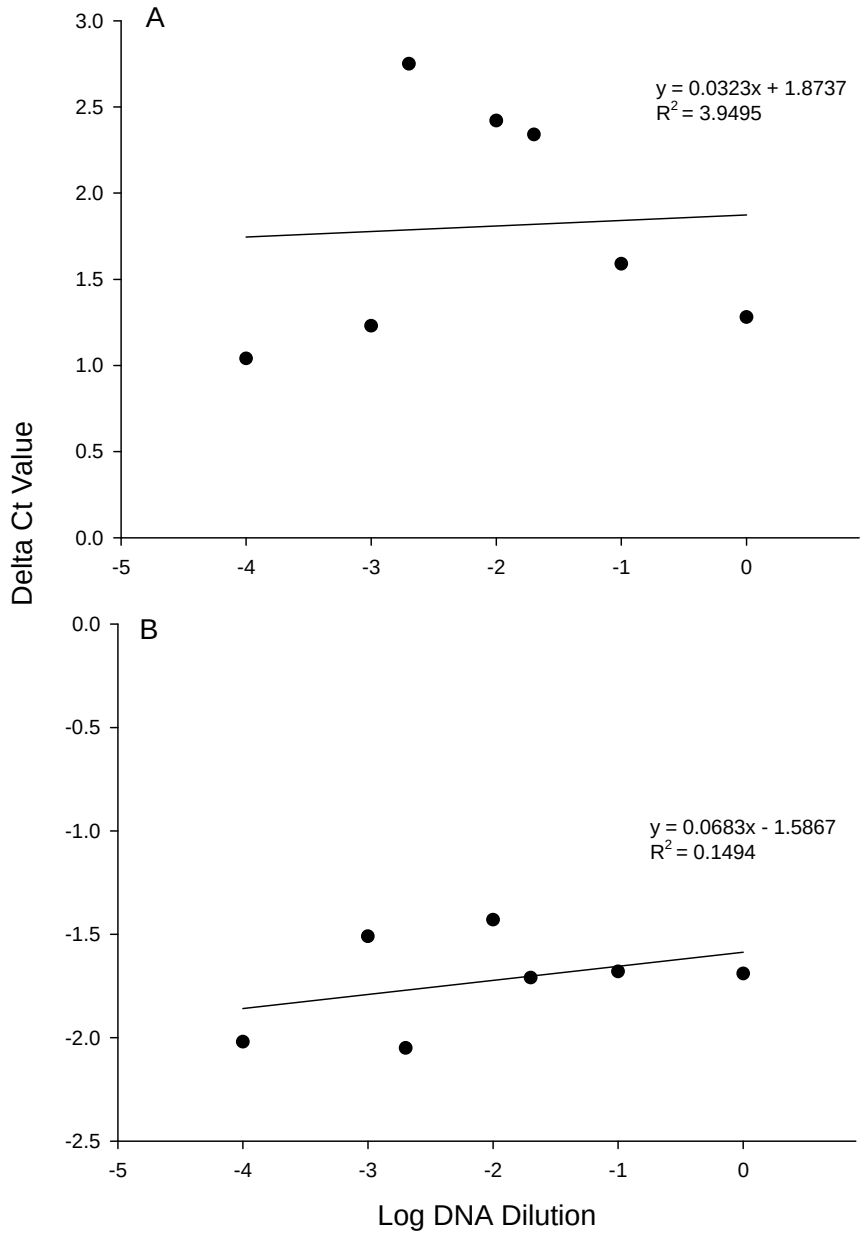


Figure 3.4: A plot of ΔCt (target-reference) to assess the amplification efficiency of the target (16S) gene and reference* (Ef1-α) *gene in samples harbouring (A) Group 1 (CABAC2) or (B) Group 2 (CABAC1) bacteria.

3.3 Results

3.3.1 Directed T-RFLP as a diagnostic approach to detect each bacterial sequence type.

The T-RFLP method was initially tested on known cabbage aphid bacterial sequence from the clone library (see Chapter 2) to confirm that each sequence type corresponded to a specific peak on the T-RFLP profile (i.e. a T-RF of a specific size). However, there were inconsistencies in the T-RFLP profiles across all bacterial sequence types in both digests. In Digest 1 the expected peaks for the T-RFs were detected for 12 of the 15 different cabbage aphid sequence types (Table 3.3A) and there were between 1 and 6 unpredicted additional peaks in most samples. The expected peaks for the T-RFs were detected in Digest 2 for 10 of the 15 cabbage aphid sequence types, although there were fewer unpredicted additional peaks (Table 3.3B). The presence of multiple unexpected T-RFs complicates the interpretation of T-RFLP results, as in unknown samples additional peaks might represent either uncharacterised types of bacteria or PCR artefacts.

Unpredicted peaks in T-RFLP profiles (Fig. 3.5) can result from background fluorescence or 'noise', and this can be corrected by setting a threshold intensity for detection determined from a dilution series. The unpredicted peaks varied in intensity over a 100-fold dilution series indicating that they were not due to background 'noise'. Another possible explanation for the additional T-RFs was incomplete digestion caused by enzyme inefficiency. Single enzyme digests indicated that unexpected peaks occurred to a similar extent among the four enzymes suggesting that enzyme inefficiency was an unlikely cause. Instead, the additional peaks most likely represented pseudo-terminal restriction fragments (pseudo-T-RFs). These occur when secondary structures form single stranded DNA amplicons during PCR amplification, creating double stranded random restriction sites within a single amplicon that generate T-RFs that are smaller in size than expected (Egert and Friedrich 2003). Pseudo T-RFs can therefore be misleading when applying T-RFLP profiles to characterise bacterial complement of cabbage aphid lines. Furthermore, it is possible that the chosen restriction sites were not sufficiently unique due to high levels of conservation in 16S sequence, or sequencing errors at restriction sites. Given that the T-RFLP method could not be optimised to detect known bacterial sequence types from a clone library real-time qPCR was considered as an alternative approach.

Table 3.3: Observed and expected T-RF sizes for each bacterial 16S sequence type based on digestion with two different sets of restriction enzymes.

A: Digest 1 *Msp*A1I/*Tsp*509I

Plasmid Sample	Expected Peak Size (bp)	Observed Peak Size (bp)	Expected Peak Detected
Cabbage Aphid Type 1	149	69,323,343,510,541	No
Cabbage Aphid Type 2	170	105, 112, **170**, 510, 564, 599	Yes
Cabbage Aphid Type 3	340	69, 100, 149, 326, **340**, 510, 542	Yes
Cabbage Aphid Type 4	350	82, **350**, 550	Yes
Cabbage Aphid Type 5	510	123, **510**	Yes
Cabbage Aphid Type 6	510	99, 417, 431, **510**, 582, 615, 662	Yes
Cabbage Aphid Type 7	510	**510**	Yes
Cabbage Aphid Type 8	80	**80**, 512, 544, 661	Yes
Cabbage Aphid Type 9	80	54, **80**, 514, 544	Yes
Cabbage Aphid Type 10	460	99, 117, 254, 431, **460**, 509, 544	Yes
Cabbage Aphid Type 11	430	100, 126, 167, 251, **430**, 420, 457	Yes
Cabbage Aphid Type 12	420	250, 526, 665	No
Cabbage Aphid Type 13	520	105, 139, 419, 431, 518, **520**, 439	Yes
Cabbage Aphid Type 14	510	104, 137, **510**	Yes
Cabbage Aphid Type 15	430	104, 137, 249	No

B: Digest 2 *Sfa*NI/*Bbs*I

Plasmid Sample	Expected Peak Size (bp)	Observed Peak Size (bp)	Expected Peak Visible
Cabbage Aphid Type 1	70	-	No
Cabbage Aphid Type 2	700	502, **700**	Yes
Cabbage Aphid Type 3	810	**810**	Yes
Cabbage Aphid Type 4	270	**270**	Yes
Cabbage Aphid Type 5	800	**800**	No
Cabbage Aphid Type 6	950	368, **950**	No
Cabbage Aphid Type 7	150	-	No
Cabbage Aphid Type 8	630	**630**	Yes
Cabbage Aphid Type 9	394	**394**, 577, 628	Yes
Cabbage Aphid Type 10	390	**390**	Yes
Cabbage Aphid Type 11	370	191, **370**	Yes
Cabbage Aphid Type 12	180	206, 368	No
Cabbage Aphid Type 13	180	368	No
Cabbage Aphid Type 14	380	-	No
Cabbage Aphid Type 15	370	**370**	Yes

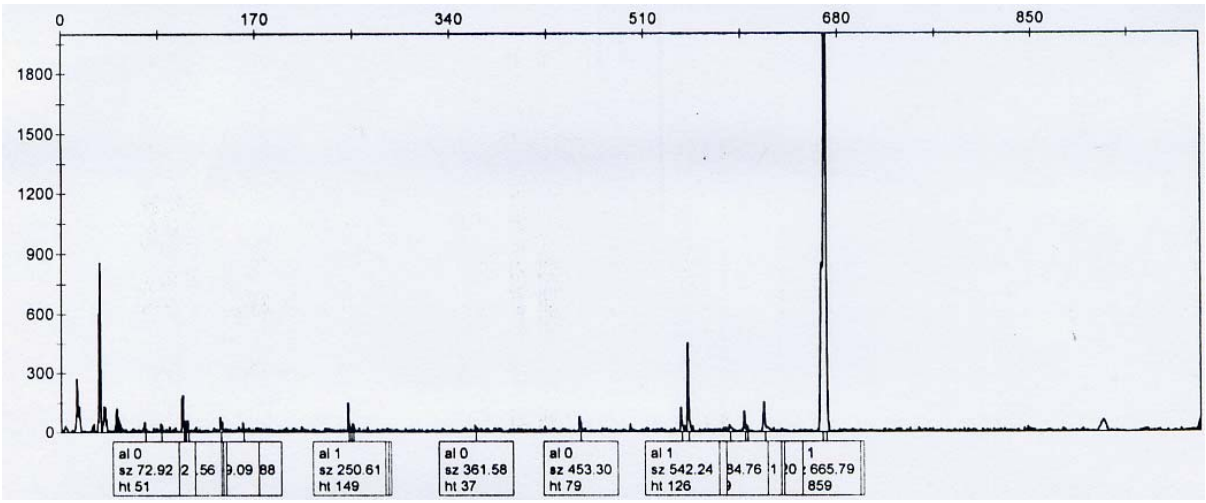


Figure 3.5: T-RFLP profile for a sample of plasmid from the clone library for cabbage aphid type 12 digested with enzymes Msp*A1I*/Tsp*509I *showing multiple unexpected peaks and no target peak (420 bp).

3.3.2 Amplification of two groups of bacteria using real-time qPCR.

3.3.2.1 Testing the real-time qPCR assay for specificity using plasmid.

To determine the specificity of the qPCR primers and probes the assays were performed on bacterial sequence types of Group 1 and Group 2 using samples of plasmid from the cabbage aphid clone library (see Chapter 2), along with the *Buchnera* 16S sequence clone (sequence type 11) and another cabbage aphid bacterial sequence type (sequence type 7). The Ct values were significantly smaller for specific compared to non-specific template assays for each probe (Table 3.4), indicating a high degree of specificity of each probe for its target sequence (One-way analysis of variance Group 1 assay (CABAC2) $F_{1,7}$ = 11.11, p = 0.021; Group 2 assay (CABAC1) $F_{1,7}$ = 42.48, p = 0.001). The qPCR assays were therefore able to detect their target bacterial sequence and non-specific amplification could be clearly differentiated. In addition, the assay designed for Group 1 bacteria did not amplify cabbage aphid sequence type 11 (*Buchnera*) or cabbage aphid sequence type 7 (Table 3.4). Also, amplification of cabbage aphid sequence type 8 (Group 1) was not as efficient as for the other bacterial types in Group 1 (9 and 10) in the Group 1 probe assay, which could be explained by suboptimal primer specificity for sequence type 8 (Appendix 2).

3.3.2.2 Application of the real-time qPCR assay to determine bacterial infection status across cabbage aphid lines.

Real-time qPCR analysis of DNA extracted from sixteen different cabbage aphid lines revealed significant differences in the relative quantity of each group of bacteria across the cabbage aphid (One-way analysis of variance Group 1 assay (CABAC2) $F_{15,42}$ = 201.33, p <0.001; Group 2 assay (CABAC1) $F_{15,47}$ = 302.45, p <0.001) (Fig. 3.6; Appendix 3). Group 1 *Pseudomonas* type bacteria and Group 2 *Erwinia* type bacteria were detected in each tested aphid line, although the relative proportion of each type varied. The aphid line with the lowest Ct value for the target gene was used as the calibrator, and this was ELC0604P for the Group 1 assay and ELC0703 for the Group 2 assay. Aphid line ELC0703 had relatively little Group 2 bacteria but relatively more Group 1 bacteria while

aphid line ELC0604P had relatively little of either type of bacteria (Fig. 3.6; Appendix 3). Post-hoc testing revealed four aphid lines that differed significantly in their bacterial complement: 1) aphid line ELC0619 which had the largest relative amount of single infection Group 2 bacteria; 2) aphid line ELC0605 which had the largest relative amount of single infection Group 1 bacteria; 3) aphid line ELC0604 which had large relative amounts of both groups of bacteria; and 4) ELC0610 which had relatively little of either type of bacteria (Fig. 3.6). There was no obvious pattern in the relative quantity of either group of bacteria that could be related to the host plant type from which the aphid line was originally collected (Fig. 3.6; Table 3.1). The intensity of the PCR products generated for the 16S-23S PCR screening of the cabbage aphid lines (Plates 2.3 A&B) was consistent in most cases with the qPCR results i.e. aphid lines with a brighter band had larger proportions of Group 1 and/or Group 2 bacteria. The 16S-23S PCR screening was carried out one year previously to the qPCR analysis indicating that secondary infection in cabbage aphid might be relatively stable over time. Additionally, the original qPCR analysis of the aphid lines (Fig. 3.6) was undertaken in August and when this was repeated for a subset of the four aphid lines used for Glasshouse Experiment 2 (Fig. 4.1) in the October of the same year the relative proportions of Group 1 and 2 bacteria in the subset of lines tested had not changed. Samples of aphids from lines ELC0606, ELC0607 and ELC0606P and ELC0604P were also repeatedly screened over a period of 18 months and found not to differ in the relative proportion of Group 1 and Group 2 bacteria, although some variation in the actual RQ values was observed.

Table 3.4: Ct values generated for each real-time assay to test specificity of the reaction.

Bacterial Sequence Type		Ct Value: Assay for Group 1 *Pseudomonas* type bacteria (CABAC2)		Ct Value: Assay for Group 2 *Erwinia* type bacteria (CABAC1)	
8		26.01		26.26	
9	Group 1	16.61	19.42	27.09	27.52
10		15.64		27.5	
12		27.23		16.31	
13	Group 2	33.95	31.58	17.42	17.25
14		29.26		20.87	
15		35.9		15.26	
7		Not detected		27.35	
11 - *Buchnera*		Not detected		25.55	

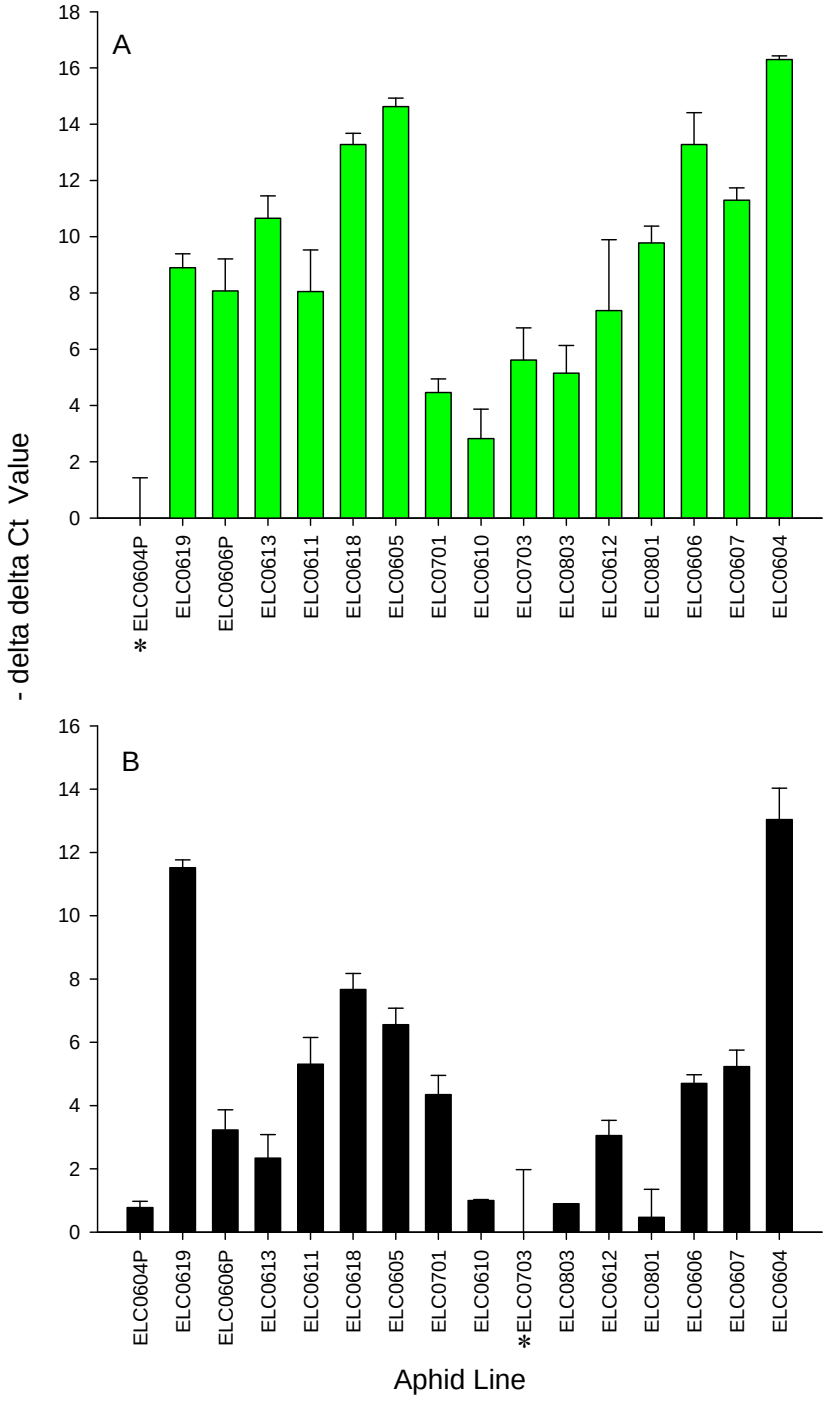


***Figure 3.6: Differences in the relative copy number of (A) Group 1 and (B) Group 2 16S sequence types (expressed as -ΔΔCt values) across fifteen different cabbage aphid lines. Error bars are +/- 95% confidence limit of the mean* (* = *aphid line used as calibrator*).**

3.4 Discussion

3.4.1 Comparison of T-RFLP and real-time qPCR methods.

Real-time qPCR gave more specific detection of target bacterial 16S sequence than T-RFLP. Method design for both techniques was complex, primarily due to the high levels of sequence conservation in the 16S gene that limited the number of unique probe binding and restriction enzyme cleavage sites. As the DNA fragment sequences cannot be retrieved and verified from T-RFLP profiles, it is essential that expected T-RFLP profiles are generated from known (cloned) sequence types. This was not the case for cloned bacterial 16S sequence from cabbage aphids, which compromised the ability of T-RFLP to determine the bacterial complement of cabbage aphids. Similar studies have also encountered problems using a directed T-RFLP approach on complex samples of DNA. The diagnostic T-RF peaks for low copy number bacteria such as *Erwinia* were masked by more common bacterial types in samples of DNA extracted from pea aphid (Haynes *et al.* 2003), while pseudo-TRFs occurred at high frequency in a study using cloned 16S genes (Egert and Friedrich 2003). The presence of many unpredicted peaks severely limited the ability of the directed T-RFLP method developed here to detect each bacterial 16S sequence type in samples of aphids. For future studies an undirected T-RFLP approach might enable differences in bacterial community composition to be determined in aphid samples rather than the presence or absence of specific bacterial types (e.g. Liu *et al.* 1997; Clement *et al.* 1998). Undirected T-RFLP has been used, for example, to successfully compare intra- and inter-specific diversity in the gut microbiota of termites (Hongoh *et al.* 2005).

Real-time Taqman® qPCR was chosen as an alternative method to detect target sequence with high specificity. Taqman® probes are capable of detecting single nucleotide polymorphisms between otherwise highly conserved sequences. The real-time qPCR assay was applied successfully to detect and determine the relative quantities of the two dominant bacterial groups, *Erwinia* and *Pseudomonas*, in samples of DNA extracted from the cabbage aphids. The real-time qPCR assay could be applied also to establish bacterial infection status in other aphid species.

3.4.2 Selection of suitable target and reference genes for real-time qPCR.

Choice of target and reference gene was critical for optimising the utility of the qPCR assay. The qPCR assay for bacteria used 16S as a target gene based on the full-length 16S gene sequence generated previously (Chapter 2). However, some bacteria possess multiple copies of the 16S gene in their genome (Klappenbach *et al.* 2000). While *E. coli* contains seven copies of the rRNA genes necessary for ribosome synthesis, many other bacteria, including *Buchnera*, have only a single copy (Shigenobu *et al.* 2000; Klappenbach *et al.* 2000). The two bacterial types that were the focus of this study, *Erwinia* and *Pseudomonas*, have approximately seven and four 16S operons per genome respectively (Fogel *et al.* 1999; McGhee *et al.* 2002). Additionally, many bacteria are polyploid. *Buchnera*, for example, exhibits extreme polyploidy and can contain hundreds of copies of the genome within each cell (Komaki and Ishikawa 1999) and it is not known whether the number of bacterial cells or the number of genome copies is physiologically more important for symbiont function. The number of genome copies inferred from the rate of amplification of the single-copy 16S rRNA gene is therefore likely to be greater than the actual number of *B. aphidicola* cells present within the aphid. Consequently, ploidy level and gene copy number can pose problems for quantitative analysis. This study determined differences in the quantity of bacterial 16S sequence rather than absolute 16S gene copy number and was suitable for comparing infection levels in a range of samples (relative to a calibrator with a low copy number). It is worth noting that *Erwinia* and *Pseudomonas* type bacteria do not exhibit polyploidy to the same extent as *Buchnera* (Klappenbach *et al.* 2000) and do not differ greatly in 16S copy number.

For absolute quantification, single copy genes are ideal targets as the number of genomes present will be proportional to the rate of target sequence amplification. While the 16S gene has been used to quantify *Buchnera*, in which it is a single copy gene (Komaki and Ishikawa 2000; Nakabachi *et al.* 2003), an alternative target gene is favoured for quantifying other types of bacteria. For example, *R. insecticola* and *H. defensa* have been quantified using the single copy gene *DnaK* as a target (Chandler *et al.* 2008) while *S. symbiotica* and pea aphid *Rickettsia* (PAR) have been quantified using the single copy genes *GroEL* and *gltA* respectively (Koga *et al.* 2003; Sakurai *et al.* 2005). Ideally, the

reference gene should be single copy for absolute and relative quantification. A number of aphid single-copy nuclear genes have been selected as reference genes including *GADPH* (Shakesby *et al.* 2009), β-tubulin (Sakurai *et al.* 2005; Shakesby *et al.* 2009) and *RPL32* (Sakurai *et al.* 2005; Shakesby *et al.* 2009). The most widely used reference gene used in aphid-symbiont studies is aphid elongation factor 1 alpha (*Ef1-α*) (Nakabachi *et al.* 2003; Oliver *et al.* 2006; Sakurai *et al.* 2005; Dunbar *et al.* 2007). While the genome of some holometabolous insects, including *Drosophila* and the honeybee *Apis mellifera*, contain two copies of the *Ef1-α* gene (Danforth and Ji 1998), *Ef1-α* in aphids is a single copy nuclear gene and therefore a suitable reference target for normalising copy numbers of bacterial genomes.

3.4.3 Alternative methods for real-time qPCR.

'Relative' and 'absolute' quantification are the two main methods of real-time qPCR (Sellars *et al.* 2007). In 'absolute' quantification methods the exact number of gene copies in a DNA sample is determined from a standard curve of Ct values generated from plasmid samples of known concentration and gene copy number (Block and Schwarz 2003; Lee *et al.* 2006). An additional method that is similar to absolute quantification but does not require the use of a reference gene estimates the number of copies of an amplified bacterial gene from a standard curve generated with serial dilutions of the amplified gene, normalised to the weight of the aphid (Douglas *et al.* 2006b; Wilkinson *et al.* 2007; Chandler *et al*. 2008). However, normalising to the weight of the aphid relies on efficient extraction of DNA, which can be determined using an aphid nuclear gene (e.g. *Ef1-α*) to relate initial aphid tissue mass to DNA content post extraction. Alternative detection methods for DNA sequences generated by real-time qPCR include SYBR® Green I technology. SYBR® Green I is a dye that intercalates with double-stranded DNA. When SYBR ® Green I is present in the reaction solution, it binds to DNA and fluoresces; the fluorescence intensity is proportional to the amount of DNA (Kubista *et al.* 2006). However, SYBR® Green I is not specific and binds to any DNA, therefore it relies on the specificity of two oligonucleotide primers to amplify the correct target DNA (Kubista *et al.* 2006). Locating specific primer sites on the highly conserved 16S gene would have

presented a significant challenge, thus SYBR® Green I was not the detection method of choice for this study.

3.4.4 Variation in relative quantity of bacteria across cabbage aphid lines forms a basis for fitness experiments.

The real-time qPCR assay revealed variation in the relative quantity of *Erwinia* and *Pseudomonas* type bacteria across the experimental cabbage aphid lines. Infection with Group 1 and Group 2 bacteria appeared to be relatively stable over the course of the study. Laboratory reared cultures are however not exposed to the same environmental pressures as wild aphid populations and it is likely that the bacteria associated with cabbage aphid are highly vulnerable to selective pressures because, unlike the commonly studied pea aphid secondary symbionts, they rely predominantly on horizontal transmission. As a consequence wild cabbage aphid populations might exhibit considerable stochastic variation in bacterial complement across any given period of time while laboratory cultures would be by nature more stable. Consequently, more detailed investigation into the stability of the bacterial associations in cabbage aphid would be worthwhile.

The variation observed in the relative quantity of *Pseudomonas* and *Erwinia* type bacteria across the experimental aphid lines could be influenced by ecological and genetic factors. The host plant from which the cabbage aphids were originally collected might influence the relative quantity of *Pseudomonas* and *Erwinia* type bacteria. Although there was no evidence for a link between bacterial complement and the collection host plant in this study, an interaction between frequencies of the secondary bacteria *R. insecticola* and aphid fitness on different host plant species has been observed in pea aphid (Leonardo and Muiru 2003; Simon *et al*. 2003; Ferrari *et al.* 2004; Tsuchida *et al.* 2004). In this study some collection plant cultivars were only represented by a single example, however, and therefore the interaction between host plant and bacterial complement in cabbage aphid requires further investigation. In addition, genotypic variation in the cabbage aphid lines might influence susceptibility to infection, leading to differences in the relative quantity of *Erwinia* and *Pseudomonas* type bacteria. Maintenance of high frequencies of *R. insecticola* in pea aphid specialists on clover is not simply related to aphid fitness or nutrition, and therefore must be linked to as yet unidentified aphid traits (Ferrari *et al.* 2006; 2007; 2008).

While pea aphid exhibits considerable population genetic differentiation throughout its range (Hawthorne and Via 2001) much less in known about cabbage aphid population genetic structure and diversity. The cabbage aphid lines used in this study were obtained from different geographical locations but it is likely that some lines exhibited higher levels of relatedness than others, which could be investigated using microsatellite markers (Caillaud *et al.* 2004). The aphid genotype x host plant species x bacterial genotype interaction requires further investigation in cabbage aphid. Differences in the relative quantity of *Pseudomonas* and *Erwinia* type bacteria on cabbage aphid fitness will form the basis for testing their effect on aphid fitness. Consequently, the real-time qPCR assay developed for this study provided a useful diagnostic tool that could also be applied to other aphid species, to increase our understanding of multitrophic interactions.

4. The effect of Group 1 and 2 bacteria on the fitness of cabbage aphid (*Brevicoryne brassicae*) and its parasitoid *Diaeretiella rapae*.

4.1 Introduction

4.1.1 Plant pathogens can have fitness consequences for their aphid host.

The majority of bacterial types characterised in the cabbage aphid (*Brevicoryne brassicae*) in Chapter 2 were γ-Proteobacteria within the orders Pseudomonales and Enterobacteriales. The phylogenetic position of the bacterial types within the two orders was used as a basis for the design of a real-time Taqman® qPCR assay (see Chapter 3) to screen cabbage aphid lines for either Group 1 *Pseudomonas* type bacteria or Group 2 *Erwinia* type bacteria. Of the two groups of bacteria, the first group comprised mainly *Pseudomonas* species similar to *Pseudomonas aeruginosa*, an opportunistic pathogen of *Drosophila* (D'Argenio *et al.* 2001), while members of the second group of bacteria detected within the cabbage aphid were highly similar to *Erwinia* species. A large number of *Erwinia* species are important plant pathogens that can cause soft rot disease in many important crops, including brassicas, by producing pectinolytic enzymes that are responsible for the disorganisation of the plant cell wall (Hugouvieux-Cotte-Pattat *et al.* 1996). In addition to being important plant pathogens, some *Erwinia* species have been shown to influence the fitness of infected insects (Harada and Ishikawa 1997; de Vries *et al.* 2004; Grenier *et al.* 2006). Infection with *Erwinia* species in western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) has both deleterious and beneficial fitness effects dependent on the diet of the host insect (de Vries *et al.* 2004). *Erwinia herbicola* (Harada *et al.* 1996), *Erwinia aphidicola* (Harada and Ishikawa 1997) and *Dickeya dadantii* (*Erwinia chrysanthemi*) (Grenier *et al.* 2006) have previously been detected in the gut of pea aphid (*Acyrthosiphon pisum*). *E. aphidicola* and *D. dadantii* (*E. chrysanthemi*) exhibit high levels of pathogenicity to pea aphid, significantly reducing survivorship and growth while *E. herbicola* has no influence on aphid fitness, indicating that pathogenicity to aphids is not a universal trait in *Erwinia* species (Harada and Ishikawa 1997; Grenier *et al.* 2006). Consequently, infection with the Group 2 *Erwinia* type bacteria might impose a fitness cost on the cabbage aphid. The extent to which plant pathogenic bacteria such as *Erwinia* species affect insect pests in arable systems is unclear, although

they could contribute to the underlying fitness effects that govern the dynamics of aphid populations in arable systems influencing not only aphid fitness but also the fitness of aphid natural enemies.

4.1.2 The secondary symbionts of aphids can influence resistance to parasitism.

Parasitoid wasps are important natural enemies of aphids; they deposit their egg in the aphid haemocoel where it develops within the living aphid until the parasitoid larva pupates and eventually kills the aphid host (Godfray 1994). Some types of symbiotic bacteria can prevent parasitoid development, allowing the aphid to recover, and are consequently key players in aphid–parasitoid interactions (Haine 2008; Oliver and Moran 2009; Oliver *et al.* 2010). To date, studies on the influence of bacteria on resistance to parasitism have focussed on the three main types of secondary symbiont bacteria characterised in the pea aphid, *Regiella insecticola*, *Hamiltonella defensa* and *Serratia symbiotica*, with little investigation into other bacteria harboured by aphids. Thus, much is now known about the positive fitness benefits attributed to the secondary symbionts, in particular *H. defensa*. The secondary symbiont *H. defensa*, a γ-proteobacterium within the Enterobacteriaceae family, dramatically increases pea aphid resistance to the parasitoids *Aphidius ervi* (Oliver *et al.* 2003; 2005; 2006; 2008) and *Aphidius eadyi* (Ferrari *et al.* 2004) when a toxin-encoding bacteriophage APSE is present (Moran *et al.* 2005c; Oliver *et al.* 2009). When *H. defensa* isolates bearing the APSE bacteriophage are present the wasp larvae dies prematurely before its development is complete and the aphid survives to develop to the adult stage and reproduce (Oliver *et al.* 2006). In the United States it is thought that 40–70% of pea aphids harbour *H. defensa*, although resistance levels vary considerably between pea aphid lines (Henter 1995; Henter and Via 1995) and between strains of *H. defensa* (Oliver *et al.* 2005). Variation in resistance is probably due to the presence or absence of the APSE bacteriophage (Oliver *et al.* 2010) and to temporal and spatial shifts in selection pressures (Gwynn *et al.* 2005). Other aphid species also harbour *H. defensa* (Haynes *et al.* 2003; Russell *et al.* 2003) including *Aphis fabae* in which it confers resistance to the parasitoid *Lisiphlebus fabarum* (Vorburger *et al.* 2009). In Chapter 2 it was shown that *H. defensa* was not present in any of the seventeen different cabbage aphid lines tested, and no other studies to date have detected *H. defensa* in *B. brassicae*.

Nevertheless, it is possible that other secondary bacteria could also influence resistance to parasitism. For example, in *Myzus persicae* from which secondary symbionts are largely absent (von Burg *et al.* 2008), one strain of the secondary symbiont *R. insecticola* found in a single *M. persicae* clone conferred high levels of resistance to the parasitoids *A. colemani* and *D. rapae* (von Burg *et al.* 2008; Vorburger *et al.* 2010a). The secondary symbiont *S. symbiotica* (Oliver *et al.* 2003) and the pea aphid X-type symbiont (aka PAXS) (Guay *et al.* 2009) have also been linked to resistance to parasitism in pea aphid.

4.1.3 Trade-offs in immunity, fecundity and survival can occur as a consequence of bacterial infection.

In laboratory populations the frequency of *H. defensa* infected aphids increased rapidly in the presence of *A. ervi* but when the parasitism pressure was removed the proportion of infected aphids decreased (Oliver *et al.* 2008). This result indicated that there was a fitness cost associated with harbouring *H. defensa* that might prevent this secondary symbiont from reaching 'fixation' in natural populations (Oliver *et al.* 2008). Some secondary symbionts, other than *H. defensa*, are known to negatively influence the fitness of their aphid hosts (Chen *et al.* 2000; Fukatsu *et al.* 2001). *M. persicae* harbouring *R. insecticola* suffers a reduction in fecundity following recovery from parasitism, again suggesting there are short term costs of resistance which have to be weighed against the long term evolutionary benefit of parasitism defence (Vorburger *et al.* 2008). Consequently, as fitness costs might prevent *H. defensa* from reaching 'fixation', variation in the susceptibility of aphid lines to parasitism is likely to be maintained by a combination of temporally and spatially fluctuating selection pressures imposed by the environment (Gwynn *et al.* 2005). Wild populations of cabbage aphid are subject to environmental fluctuations in selection pressure that could influence the effect of bacterial complement on their performance at any given time. For example, fitness costs or benefits attributable to the two groups of bacteria characterised in the cabbage aphid could have knock-on effects on the fitness of cabbage aphid parasitoids such as *D. rapae*, which attack field populations of *B. brassicae* in Scotland (Némec and Stary´ 1994; Pike *et al.* 1999). In *Drosophila*, infection with *Erwinia carotovora* activates an immune response (Basset *et al.* 2000). The parasitoid could therefore exploit aphids with immune systems compromised by infection

with pathogenic bacteria, particularly *Erwinia* species. Infection with pathogenic bacteria could also be detrimental to parasitoid embryo development dependent on the partitioning of resources from host tissues. Conversely, the reduction in fecundity caused by *Erwinia* infection would result in fewer aphid embryos, thus increasing the available resources for the parasitoid larvae. Research investigating the fitness of emergent parasitoids from insects harbouring secondary bacteria has focused mainly on the interaction between the bacteria *Wolbachia* and the fruit fly *Drosophila* in which some fitness traits of *Drosophila* parasitoids are reduced by *Wolbachia* infection (Mouton *et al.* 2004; Dobson *et al.* 2002).

There might however be numerous as yet unidentified players in the aphid–parasitoid interaction. Interestingly, plant fungal endosymbionts, that alter host plant quality by producing toxic substances, have also been shown to influence aphid tritrophic interactions (Härri *et al.* 2008; Härri *et al.* 2009). Feeding on plants infected with the fungal endophyte altered aphid metabolism, leading to increased development time of the parasitoid larva and a decrease in parasitoid lifespan (Härri *et al.* 2008: Härri *et al.* 2009). Cycles of adaptation and counter-adaptation between parasitoids and their hosts, which occur in cropped and native vegetation systems, are characteristic of the aphid–bacteria–parasitoid interactions that govern the dynamics of aphid populations (Sasaki and Godfray 1999). The aim of this study was to investigate the effect of infection with the two groups of bacteria characterised in the cabbage aphid on aphid and parasitoid fitness. Understanding the interaction between cabbage aphid, a common arable pest, its parasitoid *D. rapae*, and the bacteria associated with the aphid could shed light on a number of interesting aspects of the evolutionary dynamics of these insects as well as helping to predict their population dynamics in arable systems.

4.2 Materials and Methods

4.2.1 The aphid–parasitoid system.

The aphid–parasitoid system chosen for this study included a common pest of Scottish brassica crops, the cabbage aphid *B. brassicae*, and a generalist endoparasitoid of aphids *D. rapae*. Although there are approximately sixty aphid species that are potential hosts for *D. rapae* only five or six species are commonly attacked (Némec and Stary´ 1994; Pike *et al.* 1999). *D. rapae* shows a strong attraction to semiochemicals from crucifer plants

(Vaughn *et al.* 1996) and commonly attacks *B. brassicae* which is considered a ubiquitous host for the parasitoid. The life history strategy of *D. rapae* is typical of koinobiont aphid parasitoids. The fertilised adult female parasitoids oviposit a single egg into the body of the aphid which develops causing the exoskeleton to harden and the aphids to become 'mummies' from which an adult parasitoid emerges within 9–15 days of oviposition (Plate 4.1). *D. rapae* females are highly fecund producing up to 100 offspring during their lifetime, which under laboratory conditions is approximately two weeks (Reed *et al.* 1992). Samples of the chosen aphid–parasitoid system were collected in 2006 and 2007 from wild populations of *B. brassicae* in Tayside and Fife that were naturally parasitised by *D. rapae*.

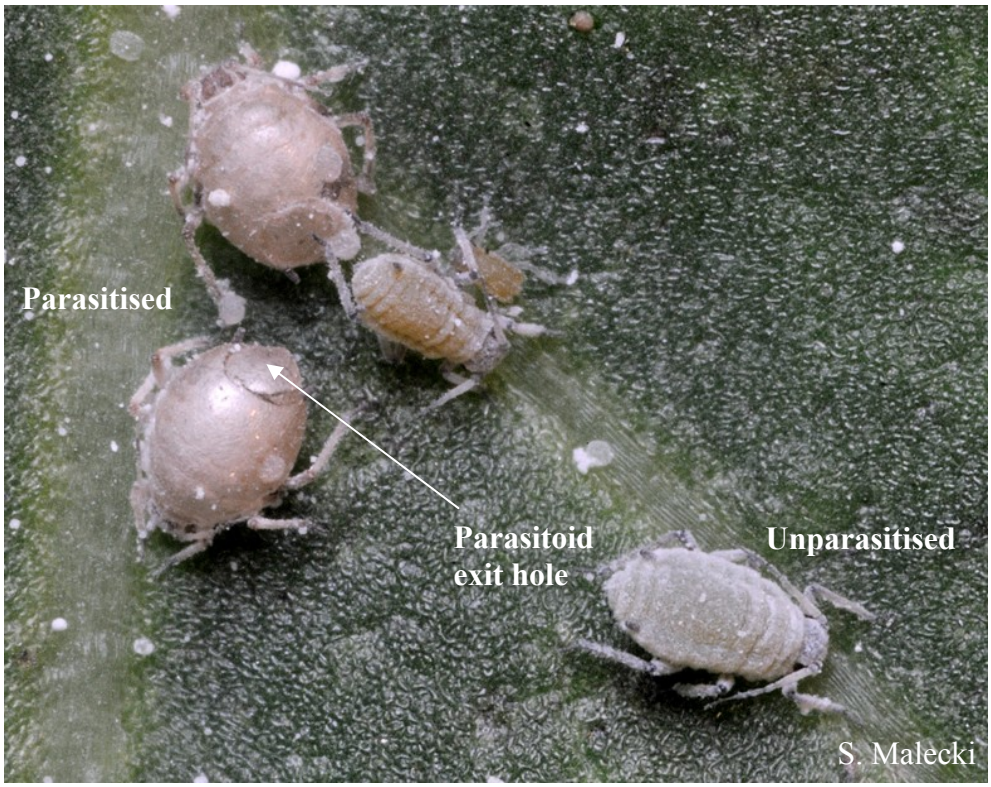


Plate 4.1: Two parasitised* B. brassicae *adults and three unparasitised nymphs.

4.2.2 Experimental Aphids.

The fitness of aphid lines with different relative densities of Group 1 and Group 2 type bacterial infection was tested in two glasshouse experiments and in a field experiment. Clonal cabbage aphid lines, derived from single parthenogenetic females collected from brassica plants in the autumn of 2006 and 2007, were used for each experiment (Section 2.2.1). The aphid lines chosen for each Glasshouse Experiment are shown in Table 4.1, which also includes details of the two aphid lines used for the Parasitism Experiment (Section 4.2.7). The bacterial infection status of the aphid lines used in Glasshouse Experiment 2 and the Parasitism Experiment were determined using the Taqman® real-time qPCR assay (Section 3.2.3) immediately prior to each experiment. For Glasshouse Experiment 1 the bacterial infection status was estimated according to the relative proportion of clones from each bacterial type generated from cloning and sequencing data.

Experimental aphids were reared on brussels sprout leaves as described previously (Section 2.2.1). Aphids for the Parasitism Experiment were bulked up on brussels sprout plants (cv. 'Evesham Special') in Perspex insect rearing culture cages (width 12 x height 14 x length 18 inches) to supply the large numbers of aphids required for this experiment.

4.2.3 Experimental Parasitoids.

Two lines of the generalist parasitoid *D. rapae*, ELC0604P parasitoids and ELC0604P parasitoids, were maintained in culture. These lines were collected from the field in August 2007 by returning to the collection site of aphid line ELC0604 (Table 4.2) and placing a brussels sprout plant infested with the aphid clone at the site. The process was repeated for aphid line ELC0606 at its respective collection site (Table 4.2). The plants were retrieved from the field after a period of ten days and the plants were caged in a controlled environment cabinet at 19°C, 50% humidity and 16h light: 8h dark, until mummies developed and wasps emerged. Emergent wasps from the parasitised material were maintained in culture on the corresponding aphid line to generate sufficient numbers of newly emergent wasps for experiments. It is important to note that since the aphids from lines ELC0604 and ELC0606 were placed in the field for parasitoid collection it could not be guarantee when they were brought back into the lab that they were the same aphids.

Consequently, the aphid lines used for parasitoid collection and maintenance were named ELC0604P and ELC0606P to indicate the difference.

Stock cultures of *D. rapae* were reared on brussels sprout plants *B. oleraceae* cv. Evesham Special enclosed in white cylindrical mesh cages (model 18/25/F Flat Top pot cage A. Johnson insect cages) planted in 7 inch black plant pots filled with insecticide free compost (William Sinclair Horticulture, Lincoln, UK, sand–perlite–peat mix containing 17:10:15 N:P:K) (Plate 4.2). Cultures were maintained at 19ºC, 18 hours light: 6 hours dark with 50% humidity in a controlled environment cabinet. The wasps were provided with a food source supplied as cotton wool balls soaked in 50% honey solution in 2 ml water filled Eppendorfs that were changed on a daily basis. Sugar concentration of honey solution can influence parasitoid behaviour and longevity, therefore the optimal concentration of 50% (Azzouz *et al.* 2004) was maintained throughout the study.

It was essential that the experimental parasitoids were of a known age. Day cohorts were established using new parasitoids emerging daily from parasitised aphid mummies attached to plant material excised from the stock cultures. On the day of emergence from the parasitised material, parasitoids were classed as zero days old and these day zero age classes were collected daily as they emerged. Care was taken to ensure that all individuals were removed from the parasitised material on each day, thus minimising cross-over of individuals between day cohorts. Each age class was kept in a plastic sandwich box, with a section removed and covered in mesh to allow airflow. Cotton wool balls soaked in 50% honey were replaced daily in each box. Once day cohorts reached seven days old the parasitoids were moved back into the stock culture.

Table 4.1: Details of aphid lines used for the glasshouse and parasitism experiments. ***ELC0606P and ELC0604P are not the same aphids as ELC0604 and ELC0606.**

Experiment	Aphid Line	Dominant Bacterial Infection
Glasshouse Experiment 1 (Pilot)	ELC0607	None
	ELC0606	Group 2 – *Erwinia* type
	ELC0701	Group 1 – *Pseudomonas* type
Glasshouse Experiment 2	ELC0610	None
	ELC0619	Group 2 - *Erwinia* type
	ELC0605	Group 1 - *Pseudomonas* type
	ELC0604	Groups 1 & 2 – Double infection
Field Experiment	ELC0610	None
	ELC0619	Group 2 - *Erwinia* type
	ELC0605	Group 1 - *Pseudomonas* type
Parasitism Experiment	ELC0604P*	None
	ELC0606P*	Group 2 - *Erwinia* type

Table 4.2: Code, collection date, location and corresponding aphid line for experimental parasitoid cultures. ***Parasitised aphid lines are suffixed with a P to differentiate them from the unparasitised aphid lines of the same name. (Grid references Landranger Ordnance Survey Map 59 1:50,000)**

Assay Name	Aphid Line	Date Collected	Location	Grid Reference
ELC0604 Parasitoids	ELC0604P*	28/08/2007	Tayport	NO457285
ELC0606 Parasitoids	ELC0606P*	28/08/2007	Letham	NO297146

***Plate 4.2: Cylinder mesh shaped cages for rearing stock cultures of* D. rapae.**

4.2.4 Bacterial complement of aphid lines.

A pilot glasshouse experiment (Glasshouse Experiment 1) was undertaken prior to the development of the Taqman® quantitative PCR assay for screening the bacterial complement of cabbage aphid lines (see Chapter 3). The bacterial complement of the aphid lines used for the pilot experiment was unknown but could be estimated from the cloning and sequencing results (see Chapter 2). The dominant bacterial infection in each aphid line was derived from the relative number of clones that generated sequence classified as either Group 1 or Group 2 bacteria (Table 4.3). At the time of sequencing aphid line ELC0606 had a larger percentage of Group 2 type bacteria while aphid line ELC0701 was dominated by Group 1 type bacteria. The aphid line that was used as a control in the first experiment was ELC0607 an aphid line in which a 16S–23S product failed to amplify indicating it harboured no bacteria other than the primary symbiont *Buchnera* (Section 2.3.2).

The density of Group 1 and Group 2 bacteria in the aphid lines used in Glasshouse Experiment 2 and the parasitism assays was characterised using the real-time Taqman® quantitative PCR molecular technique described fully in Chapter 3. The relative density of *Pseudomonas* type (Group 1 bacteria; Fig. 4.1A) and *Erwinia* type (Group 2 bacteria; Fig. 4.1B) bacteria varied across the different cabbage aphid lines and this difference was used to test the effect of bacterial complement on aphid fitness.

Table 4.3: Relative percentage of clones with Group 1 and 2 bacterial sequence type in aphid lines used in Experiment 1 (N = total number of clones sequenced).

Aphid Line	16S–23S PCR Product	Relative % of clones from bacterial group		N
		Group 1	Group 2	
ELC0606	Yes	6.70%	93.30%	120
ELC0701	Yes	76%	24%	25
ELC0607	No	n/a	n/a	n/a

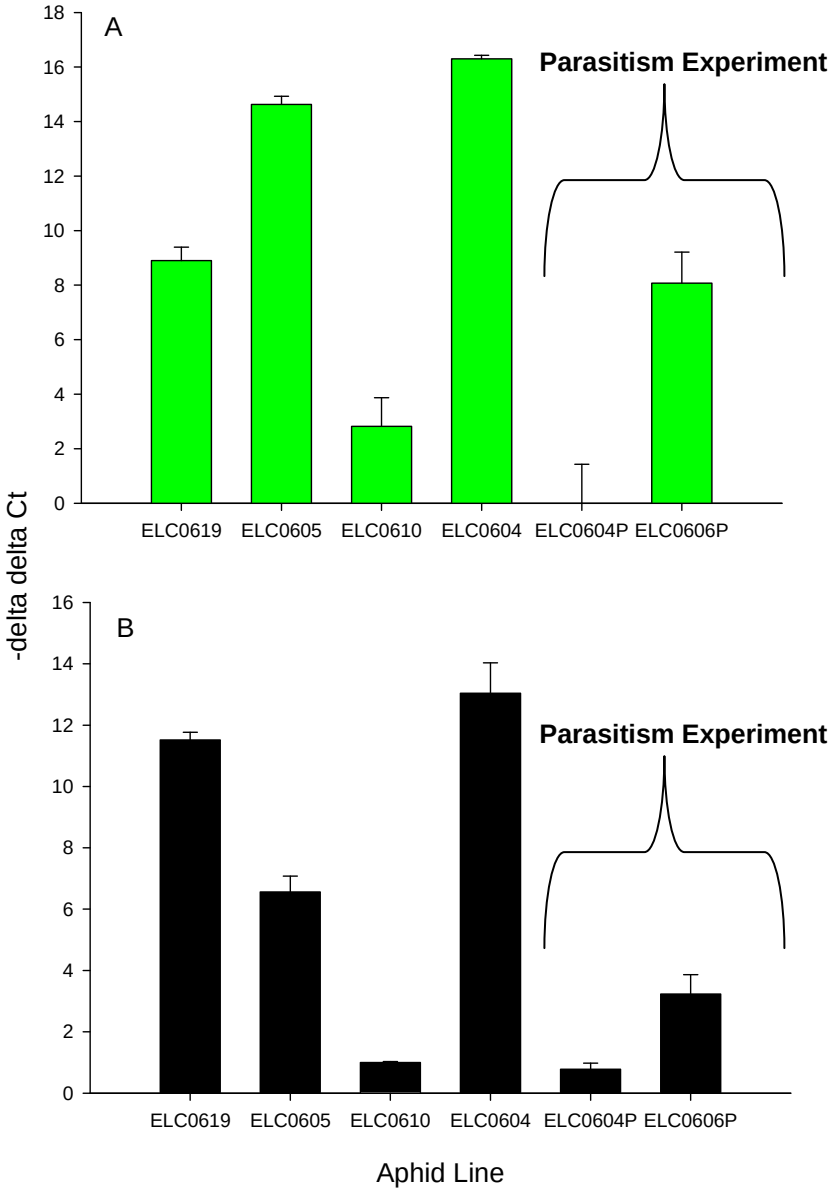


Figure 4.1: Relative differences in the number of copies of the 16S gene (expressed as -ΔΔCt values) for (A) Group 1 bacteria and (B) Group 2 bacteria across the four aphid lines used for Glasshouse Experiment 2 and the two aphid lines used for the parasitism experiment.

4.2.5 Measures of aphid fitness: glasshouse experiments.

The impact of bacterial complement on aphid fitness was tested in two separate glasshouse experiments. The first experiment was a pilot study to optimise watering regimes, plant pot sizes, the number of replicates and other variables and was conducted in June 2008 using three aphid lines. The second experiment, using the optimised conditions, was conducted in August 2009 using four aphid lines. Full details of the aphid lines used in each experiment are in Table 4.1. Two days prior to commencing the experiments, twenty reproductive adult *B. brassicae* apterae from each aphid line were selected and transferred on to fresh leaves in separate culture cups. Over the subsequent twelve hours these adults produced nymphs and were then removed and stored at -20°C in a 2 ml Eppendorf for subsequent molecular analysis of their bacterial complement, while the nymphs remained in culture. After a further twelve hours the nymphs were carefully transferred to brussels sprout seedlings using a paintbrush and caged in a 25 mm diameter mesh covered circular clip cage (Plate 4.3) with fifteen replicate plants per aphid line and three nymphs per cage. After twenty-four hours a single aphid from each clip cage was weighed to the nearest microgram on a microbalance (Sartorius SE2 Microbalance, Sartorius, Goettingen, Germany), then returned and caged to the plant and the other two nymphs in each cage were removed. Nymphal development was monitored daily until adulthood when adult weight was recorded as described above. Age at first reproduction was also recorded, after which the aphids were checked three times per week to monitor the number of offspring produced and to remove newly produced nymphs from the cage.

Two aphid fitness parameter measurements were calculated based on the data obtained from the glasshouse experiments. Firstly, the intrinsic rate of natural population increase was used as the primary method of quantifying aphid fitness and was calculated based on the formula:

Intrinsic Rate of Population Increase (R_m) = 0.738 (ln M_d) / T

(Wyatt and White 1977)

Where,

T = time from birth to initiation of larviposition (the pre-reproductive period)

M_d = number of offspring produced in the time equivalent to the reproductive period

Secondly, the relative growth rate was calculated using the formula:

RGR = [ln (final weight/initial weight)]/ time taken to reach adulthood

(Adams and van Emden 1972)

Plate 4.3: Aphid clip cage used for aphid fitness experiments.

The 4.5 inch diameter pots used in Glasshouse Experiment 1 but were deemed too small for the plant size at the end of the experiment. Consequently, larger round pots 7 inches diameter were used in the second glasshouse experiment. Brussels sprout cv. Evesham Special plants grown in insecticide free compost (William Sinclair Horticulture, Lincoln, UK, sand–perlite–peat mix containing 17:10:15 N:P:K) were used for both glasshouse experiments. Experimental plants and aphids were housed in a glasshouse heated to 18°C with 18 hours light: 6 hours dark and set up in a randomised pattern. In the pilot study the plants were watered daily with 200 ml of tap water but the plants were vulnerable to drying out as they matured. In Glasshouse Experiment 2 the volume of water was increased and the plants were watered initially with 500 ml water every second day and then daily when they became larger. At the end of the experiment, plant material was harvested and shoot fresh mass was recorded, then after oven drying at 70°C for 48 hours shoot dry mass was recorded. A sub-sample of aphids from each line was collected at the end of the experiment and stored at -20°C for subsequent analysis by real-time Taqman® qPCR assay (Section 3.2.3) to confirm their bacterial complement.

4.2.6 Measures of aphid fitness: field experiment.

A 20 m^2 plot containing 25 brussels sprout plants cv. Evesham Special (Plate 4.4) was planted in the spring of 2009 and in September a field experiment was performed to determine the fitness of aphid lines with different relative proportions of Group 1 and 2 bacteria in the field. The entire plot was contained in a mesh cage to protect from damage by birds and was weeded manually but otherwise not treated for pests.

Two days prior to commencing the experiment, twenty reproductive adult *B. brassicae* aphids from each of the three aphid lines used (Table 4.1) were transferred on to fresh leaves in separate culture cups. The adults produced nymphs during the following twelve hours and then were removed and stored at -20°C in a 2 ml Eppendorf for subsequent molecular analysis of their bacterial complement, leaving the nymphs in culture. After a further twelve hours, three of the nymphs were caged onto each brussels sprout plant in the field plot using 25 mm diameter mesh clip cages (Plate 4.3). Eight replicate plants per aphid line were used.

The nymphs were monitored daily until they reached adulthood and the age at first reproduction was recorded. Once nymphs had reached reproductive age they were checked three times per week to record number of offspring produced and to remove these nymphs from the cage. Nymph production (Md) was recorded as described for the glasshouse experiments.

Plate 4.4: Experimental plot to test aphid fitness in a field environment. Brussels sprout plants were grown in a 5x5 array in a 20m^2 plot.

4.2.7 Measures of parasitoid fitness: parasitism experiments.

The egg load and tibia length of dissected female *D. rapae* parasitoids were used as estimators of parasitoid fitness. Tibia length is commonly used as a reliable indicator of insect body mass (Nicol and Mackauer 1999), and egg load is a standard measure of parasitoid fecundity (Godfray 1994). To collect egg load and tibia length data, day cohorts of female parasitoids were established (Section 4.2.3). These day cohorts were initially established from the two parasitoid stock cultures ELC0606P and ELC0604P each of which was maintained on their respective aphid lines with significantly different relative densities of Group 1 and Group 2 bacteria (Scheffé's test Group 1 $df = 27$, $t = -18.63$, $p < 0.001$; Group 2 $df = 32$, $t = -7.81$, $p < 0.001$). After data were collected from the two initial stock cultures, a reciprocal cross of each parasitoid line with the alternative aphid line was carried out, i.e. aphid line ELC0604P was infected with parasitoids derived from aphid line ELC0606P and vice versa. Consequently, a total of four parasitism assays were performed to investigate the effect of aphid bacterial complement on parasitoid fitness.

Immediately prior to dissection, boxes containing parasitoid day cohorts were placed in a 4ºC cabinet for 20 minutes to subdue parasitoids sufficiently so that they could be handled. In the 4ºC cabinet, females were selected at random from each day cohort for dissection and placed in 2 ml Eppendorf tubes filled with 70% ethanol. Following immersion in ethanol for five minutes, parasitoids were dissected to count the number of eggs. Female parasitoids were dissected in a drop of distilled water on a microscope slide (76x26 mm, VWR International, Leuven) under a dissecting microscope (Leica dissecting microscope model XTL-101, Leica, Wetzlar, Germany) at x20 magnification. To count the egg load, dissecting pins were used to pull out the ovipositor and extract the ovaries (Mouton *et al.* 2004). Extracted ovaries were covered with a cover slip (22x22 mm, VWR International, Leuven) and lightly tapped to break the ovariole membrane and release the eggs, which were then counted at x50 magnification (Plate 4.5). The length of the parasitoid tibia was measured under x50 magnification using an eye graticule and converted into mm by calibrating with a stage graticule (x50 magnification 1 unit = 0.02 mm) (Plate 4.5). Ten individual female parasitoids were dissected from each age class from day 0 to day 7 for the first two assays, and from day 0 to day 6 for the two reciprocal assays.

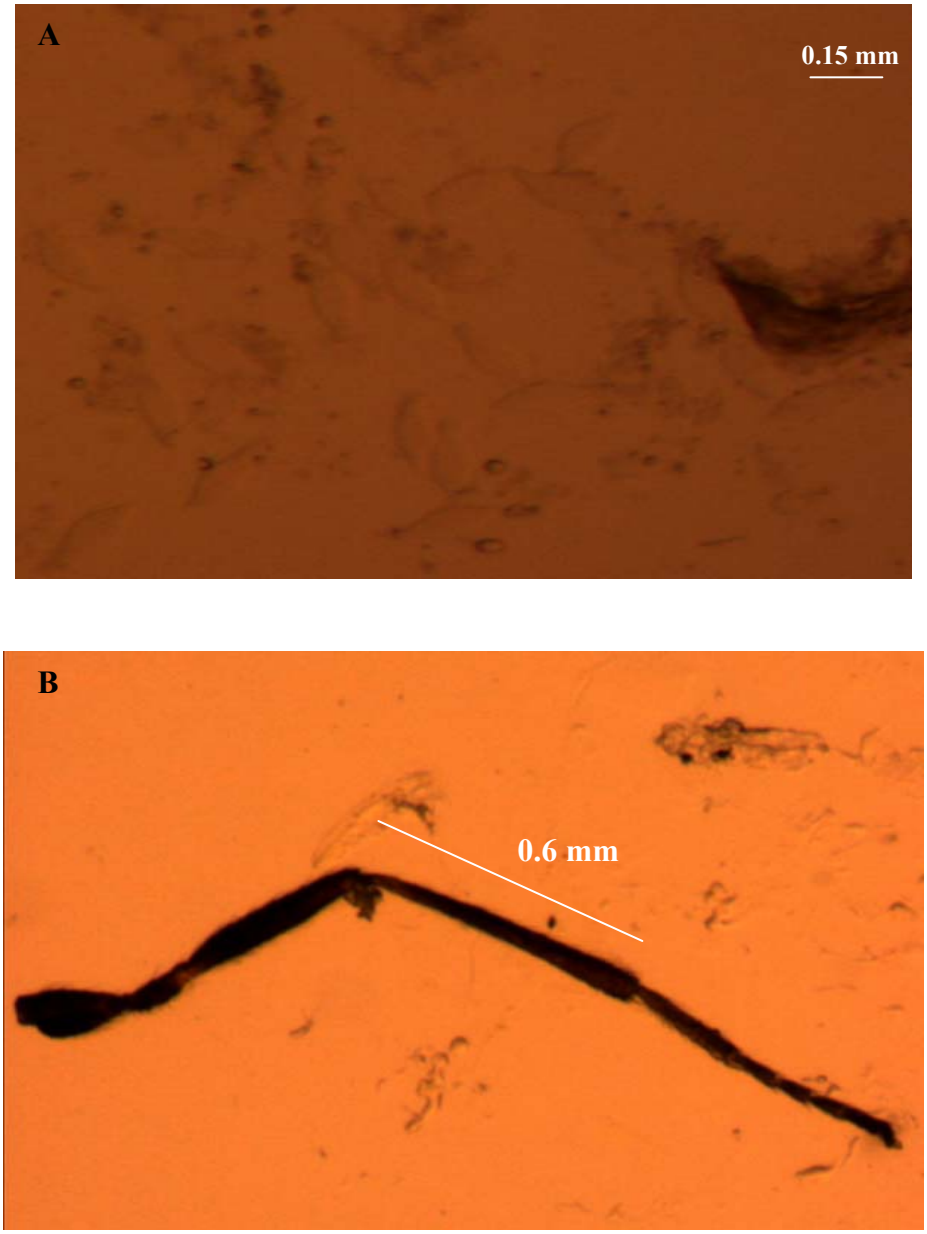


Plate 4.5: (A) **D. rapae** ***ovary dissection showing scattered eggs and (B)*** **D. rapae** ***hind leg dissected for tibia measurement (tibia is indicated) (magnification is ×50).***

4.2.8 Analysis of data from aphid and parasitoid experiments.

All statistical tests were performed using Genstat (Version 10.2). General linear models (GLM) including the variables plant fresh and dry mass, were used to determine which variables were significant in explaining the variability in the data. Differences between aphid lines in measures of aphid and parasitoid fitness were tested using one-way ANOVA followed by Scheffé's multiple comparison test to detect differences between aphid lines. Where data did not meet the criteria for normality and homogeneity of variance assumed by parametric testing then the non-parametric Kruskal–Wallis test was applied. To determine whether there was any effect of parasitoid age on the variability in egg load and tibia length two-way ANOVA was applied including age as a variable. For each aphid line the mean number of parasitoid eggs on each day was examined for the expected dynamics of an initial increase followed by a decrease with age that is common in koinobiont pro-ovigenic parasitoids (Quicke 1997). Linear regression was used to test the relationship between egg load and tibia length and thus determine whether fecundity is a function of size in *D. rapae* (Quicke 1997).

Survival of each aphid line was assessed by survivorship analysis using GLIM (Aitken *et al.* 1989). Individual aphids were monitored and time of death recorded and assigned a value of zero, individuals that survived the period of the experiment to an unknown time in the future were 'censored' and assigned a value of one. It is important to note that this method of assigning censoring values applies to GLIM in Genstat using the RSURVIVAL function and may differ for other versions of GLIM. Survivorship plots were generated using the Kaplan–Meier estimate of the survivor function (Kaplan and Meier 1958), which takes into account 'censored' data to compare survival across the different aphid lines. The survivor function shows the proportion of initial individuals still alive at time t in the experiment, has an intercept of 1 (i.e. all individuals are alive at time 0) and shows the probability of surviving longer than time t. To compare the survival curves for each aphid line a parametric log linear hazard model (Crawley 1993) was fitted to the censored data (response variable). A Weibull distribution model with a Poisson error distribution, a log link function and $\alpha\log(t)$ as an offset was deemed the most appropriate model and was generated using the RSURVIVAL function in Genstat (Aitken *et al.* 1989). In each model α represents the shape parameter (Crawley 1993). The exponential model,

another commonly used survivorship model, assumes the hazard function is independent of age ($\alpha = 1$), while the Weibull model includes other values of the hazard function ($1>\alpha>1$), thus allowing the death rate to increase or decrease with age, as well as having the exponential as a special case, and is therefore more ecologically sound (Crawley 1993).

4.3 Results

4.3.1 Glasshouse experiments showed aphid fitness varied with bacterial complement.

The fitness of aphid lines with different bacterial complements was compared in two glasshouse experiments. In both experiments the intrinsic rate of population increase (Rm) correlated with aphid relative growth rate (RGR) (Experiment 1 $r = 0.6349$, $df = 25$, $p<0.01$ and Experiment 2 $r = 0.4722$, $df = 41$, $p<0.01$), confirming that cabbage aphids with a faster growth rate produced more offspring (Dixon 1973). In both experiments, the relative growth rate did not vary between aphid lines (Experiment 1 Kruskal–Wallis one-way analysis of variance $H_{2,27} = 0.9841$, $p = 0.611$ and Experiment 2 Kruskal–Wallis one-way analysis of variance $H_{3,48} = 0.4265$, $p = 0.935$), indicating that bacterial complement did not affect this measure of aphid fitness (Fig. 4.2 B&D). In Experiment 1, the intrinsic rate of population increase (Rm) differed significantly between aphid lines (Kruskal–Wallis one-way analysis of variance $H_{2,29} = 6.065$, $p = 0.048$). The mean Rm for aphid line ELC0606 (mean = 0.195) which harboured relatively more Group 2 type bacteria was significantly smaller than the mean Rm for the aphid lines that had either no infection with either group of bacteria (ELC0607 = 0.256) or relatively more Group 1 type bacteria (ELC0701 = 0.260) indicating that harbouring relatively more Group 2 type bacteria caused a fitness cost to the aphid in terms of reproductive output (Fig. 4.2 A). However, in Experiment 2 Rm did not differ significantly between the four aphid lines tested (Kruskal–Wallis one-way analysis of variance $H_{2,44} = 1.112$, $p = 0.774$), although the aphid line ELC0619 harbouring a greater density of Group 2 bacteria exhibited the lowest values of Rm (mean Rm = 0.223) compared to the other aphid lines (Fig. 4.2 C). In fact the sample size for aphid line ELC0619 was the smallest ($N = 8$) for comparing Rm and RGR as many of the ELC0619 aphids died prior to the start of the reproductive period.

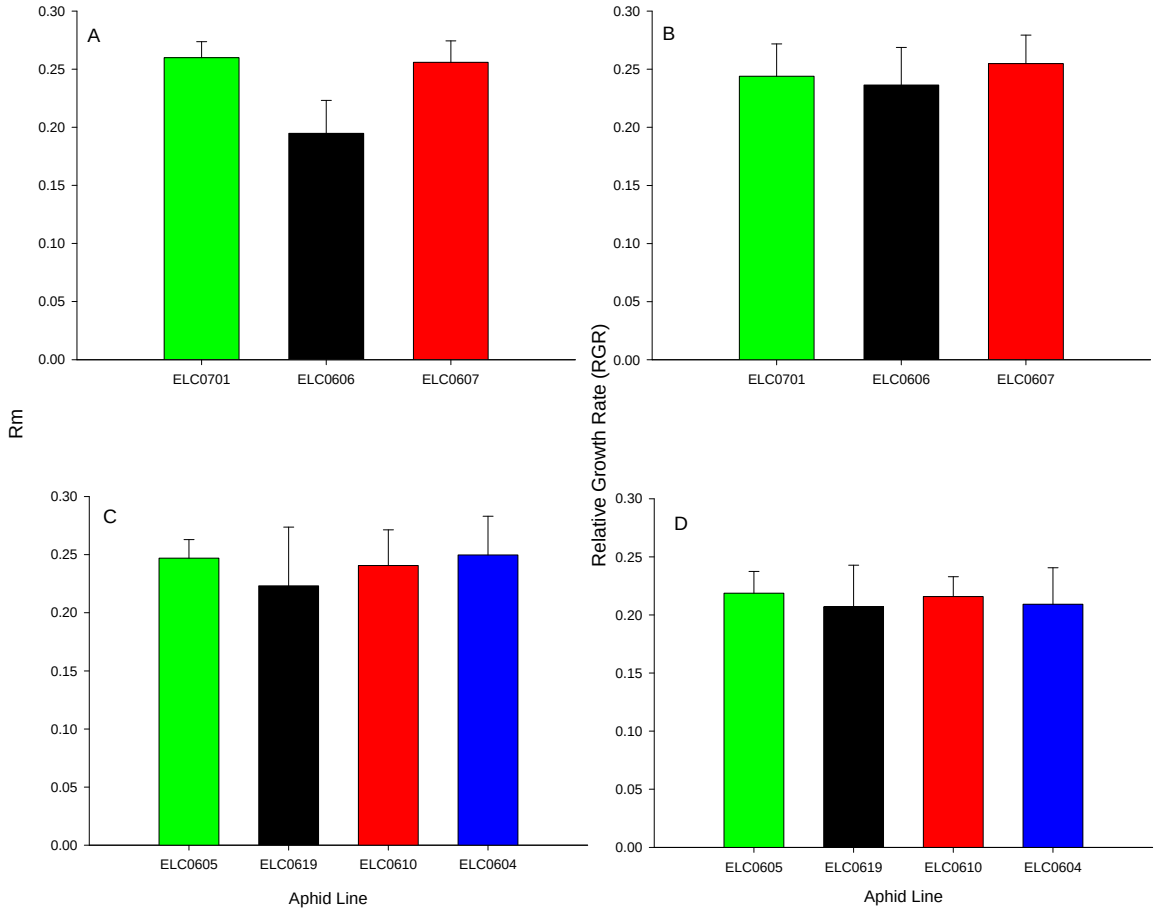


Figure 4.2: Mean intrinsic rate of population increase (Rm = A, C) and relative growth rate (RGR = B, D) (values are means +/- 95% Confidence Interval) for aphid lines in Glasshouse Experiments 1 (A&B) and 2 (C&D) (Green = Group 1 infection, Black = Group 2 infection, Red = no dominant infection, Blue = double infection).

4.3.2 Survival of aphid lines with different bacterial complements in the glasshouse.

Plots of the Kaplan–Meier survival function for the different aphid lines indicated that there were significant differences in aphid survival in both the Glasshouse Experiments 1 and 2 (Fig. 4.3 A&B) (Log-rank test Experiment 1 $T_{2,41} = 7.926$, $p = 0.019$ and Experiment 2 $T_{3,60} = 12.810$, $p = 0.005$).

To determine which aphid lines exhibited significant differences in survival, the aphid line associated with the most isolated dissimilar Kaplan–Meier plot was chosen as a candidate reference line to fit a Weibull distribution model in GLIM to the censored data. In Experiment 1 aphid line ELC0701, which harboured a greater density of Group 1 type bacteria, had a significantly higher survivorship potential than either the aphid line with a greater density of Group 2 type bacteria (ELC0606) or the aphid line that harboured relatively little of either group of bacteria (ELC0607) (Table 4.4). Aphid lines ELC0606 and ELC0607 did not differ significantly from each other which indicated that harbouring Group 1 type bacteria had a positive impact on aphid survival compared to harbouring Group 2 type bacteria or relatively little of either type of bacteria. In Experiment 2, aphid line ELC0619, with a greater density of Group 2 type bacteria, again exhibited a significantly lower survivorship potential than the aphid line with a greater density of Group 1 type bacteria (ELC0605) and also performed poorly compared to the aphid line that harboured both types of bacteria (ELC0604) (Table 4.4). Interestingly, the aphid line that harboured relatively little of either type of bacteria (ELC0610) did not differ significantly in survival potential from either ELC0619 (Group 2 infection) or the other two aphid lines (Table 4.4). Consequently, the results from Experiment 2 were in agreement with Experiment 1 that there was a positive effect of harbouring Group 1 type bacteria on aphid fitness compared to harbouring Group 2 alone. It should be noted that Experiment 1 was a pilot study for Experiment 2, and as there was no quantification of the bacterial complement of the aphids used in the pilot experiment, bacterial complement was estimated from sequencing data. Thus, the results of Experiment 1 are included for completeness, but should be treated with caution.

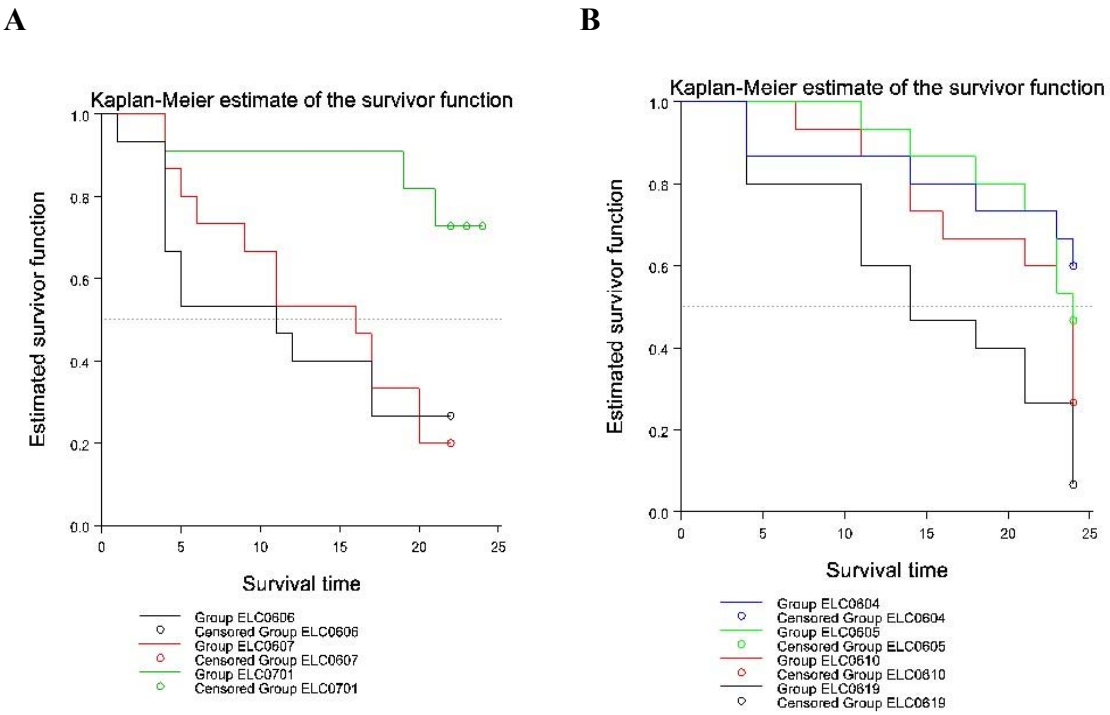


Figure 4.3: Kaplan–Meier estimate of the survival function, representing the proportion of deaths between days 0 and 25 of the experiments, for aphid lines with different bacterial complements compared in (A) Experiment 1 and (B) Experiment 2 (Green = Group 1 infection, Black = Group 2 infection, Red = no dominant infection, Blue = double infection).

Table 4.4: Results for parametric comparison of survivorship of aphid lines with different bacterial complements using GLIM to fit a parametric model with a Weibull distribution to the censored data.

Experiment	Aphid Line	Bacterial infection	Df	t-statistic	p-value	Significance
1	ELC0701	Group 1	2	Reference		
	ELC0607	None	2	2.45	<0.05	*
	ELC0606	Group 2	2	2.6	<0.05	*
2	ELC0619	Group 2	3	Reference		
	ELC0610	None	3	-1.68	>0.05	Not significant
	ELC0605	Group 1	3	-2.56	<0.05	*
	ELC0604	Groups 1&2	3	-2.81	<0.05	*

4.3.3 Fitness and survival of aphid lines with different bacterial complements in the field.

There was no significant difference in the intrinsic rate of population increase (Rm) across aphid lines with different bacterial complements when their fitness was compared in the field (Fig. 4.4A) (Kruskal–Wallis one-way analysis of variance $H_{2,19} = 0.9309$, $p = 0.6228$). The survivorship of the different aphid lines was also not significantly different in the field (Log-rank test, $T_{2,24} = 1.582$, $p = 0.453$) (Fig. 4.4B).

It should be noted that during the Field Experiment the experimental aphids experienced periods of heavy rain, wind and low temperatures that would be likely to have had a greater impact on aphid fitness than bacterial complement alone.

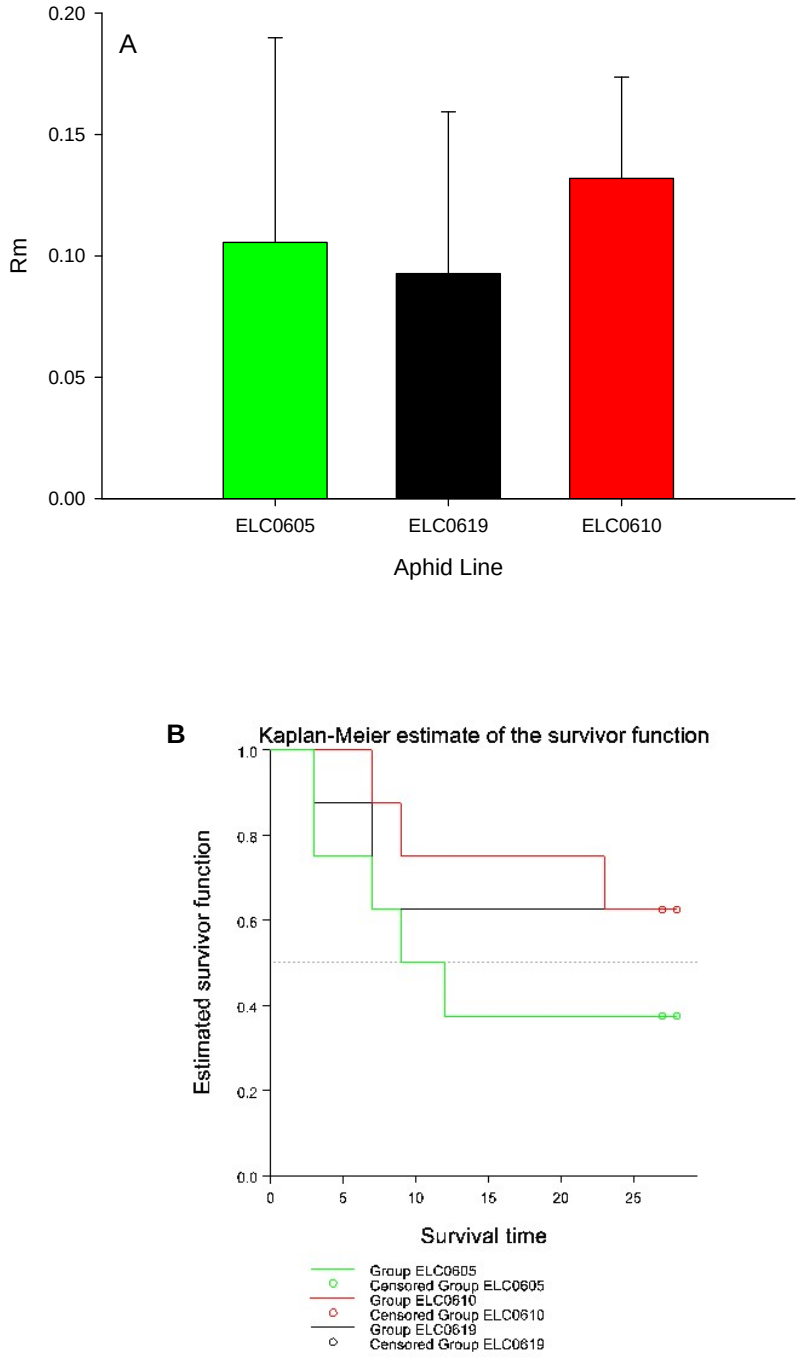


Figure 4.4: (A) Mean Rm for aphid lines with different bacterial complements (Green = Group 1 infection, Black = Group 2 infection, Red = no dominant infection) (values are means +/- 95% Confidence Interval) and (B) Kaplan–Meier estimate of the survival function compared in the Field Experiment (Green = Group 1 infection, Black = Group 2 infection, Red = no dominant infection).

4.3.4 Fitness of emergent parasitoids from aphid lines with different bacterial complements.

4.3.4.1 Egg load in day cohorts.

There was a significant effect of the age of parasitoids on their egg load. Since *D. rapae* is koinobiont and pro-ovigenic, mean egg load would be expected to decrease with parasitoid age as pro-ovigenic parasitoids species are born with a full complement of eggs that are depleted over time. Largest egg loads were associated with one-day-old parasitoids while the smallest egg loads were recorded in six-day-old parasitoids. However, there was no consistent trend with parasitoid age, which may relate to the fact that the parasitoids were not exposed to aphids and the only mechanism for reduction in egg load was reabsorption (Fig. 4.5).

4.3.4.2 Relationship between egg load and parasitoid size.

Fecundity does not appear to be a function of size in *D. rapae* as parasitoids with a longer tibia did not carry larger numbers of eggs. The relationship between egg load and tibia length was not significant in any of the parasitism assays (Fig. 4.6), (Simple Linear Regression, ELC0604P Aphids ELC0604P Parasitoids $R^2 = 2.2$, $F_{1,86} = 2.94$, $p = 0.09$; ELC0606P Aphids ELC0606P Parasitoids $R^2 = 1.6$, $F_{1,85} = 0.62$, $p = 0.443$; ELC0606P Aphids ELC0604P Parasitoids $R^2 = 0.6$, $F_{1,48} = 1.27$, $p = 0.265$; ELC0606P Aphids ELC0606P Parasitoids $R^2 = 1.5$, $F_{1,48} = 1.73$, $p = 0.195$). In the reciprocal cross ELC0604 aphids with ELC0606P parasitoids the variability in the data was markedly reduced (Fig. 4.5D and 4.6D), potentially indicating a change in the relationship between parasitoid fitness and aphid bacterial complement that was reflected in the egg load and tibia length of emergent parasitoids.

4.3.4.3 Impact of aphid bacterial complement on parasitoid egg load and tibia length.

There was a significant difference in the egg load of parasitoids emerging from the different experimental aphid lines (One-way analysis of variance, $F_{3,270} = 29.75$, $p < 0.001$). Post hoc testing using Scheffé's test revealed significant differences in egg load between all

parasitism assays except the two reciprocal crosses (Fig. 4.7A). The mean egg load was significantly smaller in emergent parasitoids from aphid line ELC0604P (mean egg load = 49), which harboured relatively little of either type of bacteria, than in parasitoids emerging from aphid line ELC0604P (mean egg load = 68), which harboured a greater density of Group 2 bacteria. In addition, the egg load of the reciprocal crosses (mean egg load = 83 and 89) was significantly larger than the uncrossed assays. The tibia length of parasitoids emerging from the different experimental aphid lines also varied significantly (Fig. 4.7B) (One-way analysis of variance, $F_{3,270} = 4.49$, $p = 0.004$). However, post-hoc analysis using Scheffé's test that revealed the only significant difference in tibia length occurred between the uncrossed assay with ELC0604P aphids and parasitoids and the reciprocal cross between ELC0606P aphids and ELC0604P parasitoids. The mean tibia length was smaller in emergent parasitoids from the uncrossed ELC0604P assay (mean tibia length = 0.60 mm), than for parasitoids emerging from the reciprocal cross ELC0606P aphids with ELC0604P parasitoids (mean tibia length = 0.65 mm) indicating that exposure to aphids harbouring Group 2 bacteria had a positive effect on the size of emergent parasitoids previously cultured on aphids with relatively little of either type of bacteria. The results of the parasitism experiment suggest that parasitoid size benefits when parasitoids are exposed to aphids harbouring Group 2 bacteria, while egg load is improved specifically on Group 2 aphids and generally on both reciprocal crosses (Fig. 4.7 A&B). Counter-adaptation of parasitoids to novel hosts could account for this trend as the new niche available to the parasitoids on the reciprocal crosses could promote increased size and fecundity.

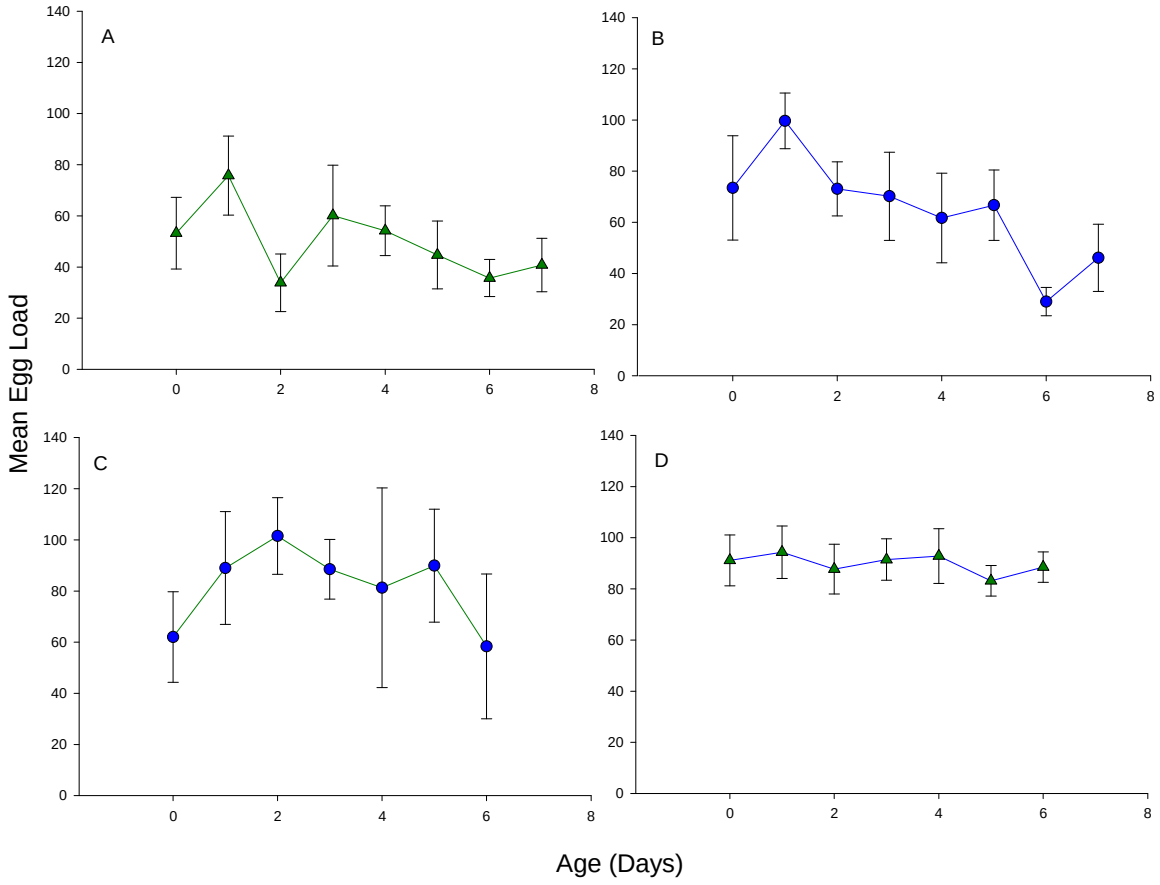


Figure 4.5: Variation in mean egg load with age (values are mean +/- 95% Confidence Interval) in emergent parasitoids from each of the four parasitism assays. A = ELC0604P aphids with ELC0604P parasitoids, B = ELC0606P aphids with ELC0606P parasitoids, C = ELC0606P aphids with ELC0604P parasitoids, D = ELC0604P aphids with ELC0606P parasitoids.

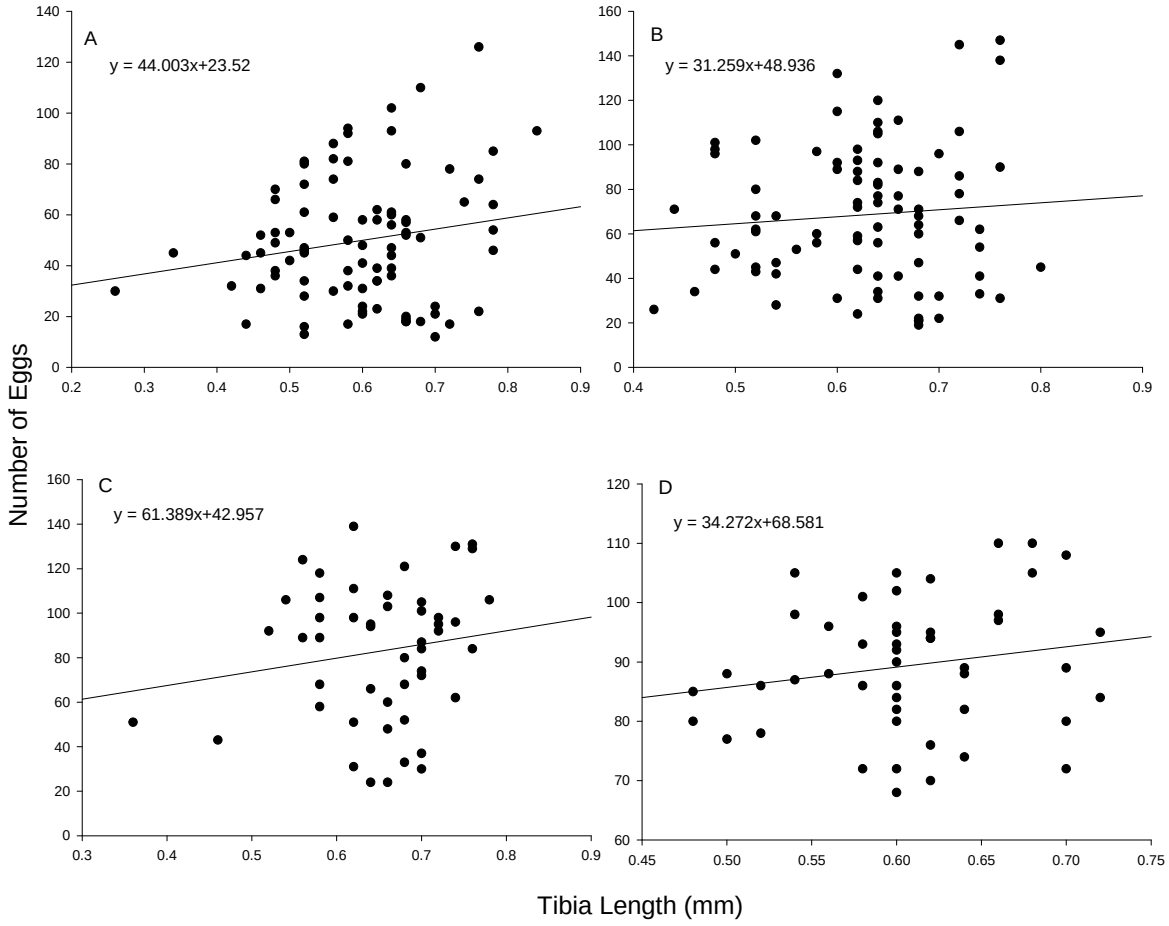


Figure 4.6: Egg load versus tibia length for each parasitism assay (equation represents regression line). A = ELC0604P aphids with ELC0604P parasitoids, B = ELC0606P aphids with ELC0606P parasitoids, C = ELC0606P aphids with ELC0604P parasitoids and D = ELC0604P aphids with ELC0606P parasitoids.

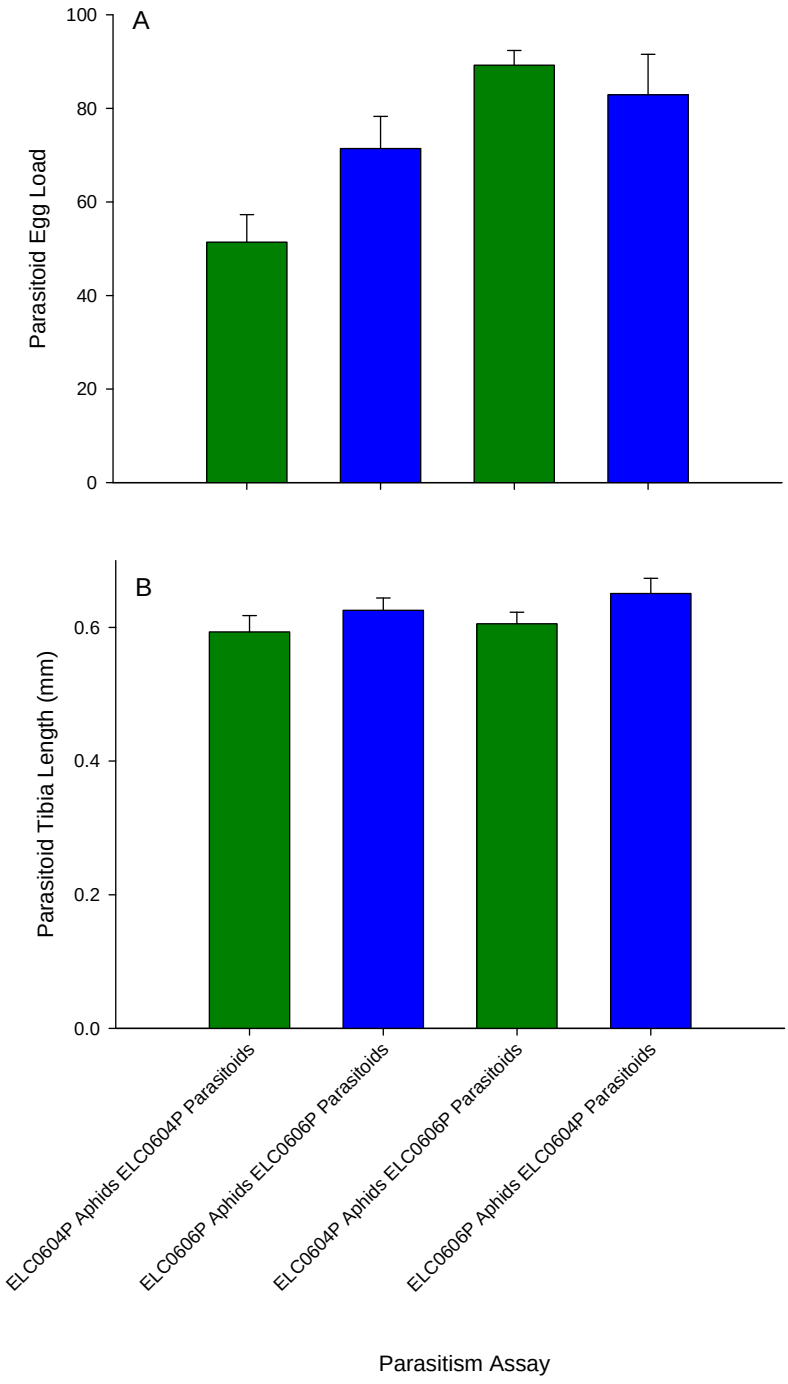


Figure 4.7: (A) Egg load and (B) tibia length (values are mean +/- 95% Confidence Interval) of emergent parasitoids from aphid lines with either Group 2 bacterial infection (ELC0606P) (Blue) or relatively little of either infection (ELC0604P) (Green).

4.4 Discussion

4.4.1 Fitness effects were associated with harbouring the bacteria characterised in the cabbage aphid.

This study investigated whether infection with Group 2 *Erwinia* type bacteria and Group 1 *Pseudomonas* type bacteria had an impact on the fitness of cabbage aphids and their parasitoid to identify potential fitness trade-offs. In pea aphid various beneficial fitness effects, including increased resistance to parasitism, have been attributed to the presence of facultative secondary bacteria (Montllor *et al.* 2002; Oliver *et al.* 2003; Ferrari *et al.* 2004). The secondary symbionts harboured by the pea aphid are maternally transmitted and are maintained at stable levels in laboratory aphid populations. Recent evidence suggests secondary symbiont infection frequencies in pea aphid populations are maintained by positive and negative pressures resulting from fitness trade-offs due to harbouring the symbiont (Oliver *et al.* 2008). At the outset of this study it was unclear if the bacterial types characterised in the cabbage aphid would have any effect on the fitness of the aphid or the parasitoid and thus whether they would be maintained in cabbage aphid populations due to positive selection. The bacteria characterised in the cabbage aphid are likely to reside in the aphid gut, unlike the secondary symbiont bacteria of the pea aphid, and as a consequence vertical transmission from one generation to the next is likely to be less stable. There are however a number of examples of stable beneficial gut microbe–host symbioses (Dillon and Dillon 2004). The maternally transmitted gut symbionts of stinkbugs, for example, are essential for nymphal growth and development (Fukatsu and Hosokawa 2002; Hosokawa *et al.* 2006), and the hind-gut symbionts of termites play a role in the enzymatic degradation of cellulose, providing nutrients for their host (Tokuda and Watanabe 2007). However, in both of these cases symbiotic bacteria reside in specialised gut structures (Fukatsu and Hosokawa 2002; Hosokawa *et al.* 2006; Tokuda and Watanabe 2007). While these examples of gut symbionts are non-pathogenic, the presence of other bacteria in the insect gut, including *Erwinia* and *Pseudomonas* species, can have deleterious pathogenic effects on the aphid host (Harada and Ishikawa *et al.* 1997; D'Argenio *et al.* 2001; Grenier *et al.* 2006). This study confirms the findings of previous studies (Harada

and Ishikawa 1997; Grenier *et al.* 2006) that some *Erwinia* species have pathogenic effects on aphids. In addition this study indicates that bacterial infection might improve parasitoid fitness on cabbage aphid rather than increasing aphid resistance to parasitoid wasps.

4.4.2 Dynamics of infection with **Erwinia** *species in aphids.*

Based on the standard index for estimating aphid fitness, 'intrinsic rate of population increase' (Rm) (Wyatt and White 1977), Glasshouse Experiment 1 indicated that there was a negative effect of Group 2 *Erwinia* type infection on aphid fitness though this trend was not observed in Glasshouse Experiment 2. There were, however, low numbers of aphids surviving to reproductive age in Glasshouse Experiment 2 and consequently survival analysis, which is particularly useful for analysing data from aphid fitness experiments in which individuals die during the course of the experiment, was chosen as an alternative fitness measure (Ma 2009). Survival analysis indicated that aphid performance was significantly improved in the presence of Group 1 *Pseudomonas* type bacteria compared with Group 2 *Erwinia* type bacteria infection or infection with little of either type of bacteria. Previous studies also found that some species of *Erwinia* were pathogenic to aphids, dramatically reducing survival, fecundity and body weight (Harada and Ishikawa 1997; Grenier *et al.* 2006) while other *Erwinia* species, such as *E. herbicola* and *E. rhapontici*, had no effect on aphid fitness (Harada and Ishikawa 1997; Grenier *et al.* 2006). The results of this study indicate a degree of pathogenicity of *Erwinia* type bacteria towards the cabbage aphid host. However, it should be noted that previous studies used artificial infections by injection or feeding to determine the effect of *Erwinia* infection on aphid fitness in contrast to the natural infections in the current study. Ingestion of just ten *E. chrysanthemi* cells was lethal to pea aphid (Grenier *et al.* 2006) which the authors claim would be relevant to natural levels of *Erwinia* infection in plant tissues (Toth *et al.* 2003). Aphids could acquire *Erwinia* from sampling cells of the plant vascular tissues, including the phloem upon which they feed or the xylem from which they can ingest sap for long durations under variable stress conditions (Spiller *et al.* 1990). It is possible that they could also acquire *Erwinia* through contact with the honeydew of infected aphids or contaminated leaf surfaces. Thus, there are several potential sources of *Erwinia* infection for aphids, although the extent to which aphid populations are naturally infected with *Erwinia* is

unclear. *Erwinia* species were not detected in a survey of 324 pea aphid populations (Haynes *et al.* 2003) while this study suggested that *Erwinia* infection is relatively common in cabbage aphid, although often at low densities. Consequently, the profoundly pathogenic effects of *Erwinia* infection observed in artificially infected pea aphids are unlikely to occur at the low infection densities observed in natural aphid populations.

The high levels of aphid mortality recorded in previous studies could also have been a consequence of an imbalance in the bacterial population caused by feeding *Erwinia* species to aseptic aphids (Harada and Ishikawa 1997). Some bacteria can produce antibiotics that interfere with the normal biological functions of other bacteria. *Bacillus thuringiensis* for example suppresses the quorum-sensing-dependent virulence of *Erwinia carotovora* (Dong *et al.* 2004). Consequently, other members of the Enterobacteriaceae co-inhabiting the aphid gut could suppress the pathogenic effects of *Erwinia* species in naturally infected aphids. Insects with simple digestive tracts such as aphids harbour a lower diversity of bacteria than insects with more complex gut structures (Dillon and Dillon 2004). Estimates of the diversity of bacteria in the aphid gut range from only a few types, up to seven different taxonomic groups, which exhibit high levels of temporal variation as new types are acquired from the environment and other types are lost (Douglas 1988; Grenier *et al.* 1994; Harada *et al.* 1996). Given the dynamic nature of bacterial infection in the aphid gut, the potential for interaction between bacteria is high (Dillon and Dillon 2004). The results from Chapter 3 suggested that double infections with both Group 1 *Pseudomonas* type bacteria and Group 2 *Erwinia* type bacteria are common in cabbage aphid. This study recorded a positive effect of harbouring Group 1 type bacteria on aphid fitness compared to harbouring Group 2 alone indicating that infection with Group 1 *Pseudomonas* bacteria might reduce the pathogenicity of the Group 2 *Erwinia* type bacteria.

4.4.3 Bacterial infection influences the fitness of emergent parasitoids.

Aphid secondary bacteria can influence aphid resistance to parasitism. The secondary symbiont *H. defensa* dramatically increases pea aphid resistance to parasitism (Oliver *et al.* 2003; 2005; Ferrari *et al.* 2004) and *R. insecticola* has a similar effect in *M. persicae* and *A. fabae* (von Burg *et al.* 2008; Vorburger *et al.* 2010a). The mechanism of

resistance in pea aphid has recently been linked to the toxin-encoding APSE lysogenic lamdoid bacteriophage (Moran *et al.* 2005c; Oliver *et al.* 2009), which contains homologues of a protein known to target eukaryotic tissue. Different phage variants confer different levels of resistance to parasitism (Degnan and Moran 2008a&b; Degnan *et al.* 2009). The APSE bacteriophage has not been linked to resistance attributed to *R. insecticola* but another phage variant could be causing the resistance effect (Vorburger *et al.* 2010a). Bacteriophages are known to carry key virulence factors for many pathogenic bacteria. In *E. coli* a bacteriophage called Shiga toxin (*stx*) encodes virulence factors (O'Brien *et al.* 1984). Although it is not known whether *B. brassicae* harbours bacteriophage-mediated resistance to parasitism, the genome of *E. chrysanthemi* contains four genes encoding homologues of insecticidal toxins, which might influence the pathogenicity of the bacterium and consequently aphid fitness and defence against parasitism (Grenier *et al.* 2006). Homologues of the toxin encoding genes in *E. chrysanthemi*, designated the *cyt* family, are found only in the Gram-positive bacteria *Bacillus thuringiensis* which is an entomopathogen used to control insects and nematodes (Guerchicoff *et al.* 2001). Toxicity of these genes occurs when products of the *cyt* genes, which form cytolytic pores in the gut membrane, are released by the ingested bacteria and lead to bacterial invasion of the insect, compromising its survival (Li *et al.* 1996; Promdonkoy and Ellar 2000). Infection with the Group 2 *Erwinia* type bacteria in this study had negative effects on aphid fitness suggesting that the bacterium might compromise cabbage aphid immunity and consequently its defense against parasitism.

If the ability of the cabbage aphid to resist parasitism was compromised by infection with pathogenic *Erwinia* type bacteria then an increase in the fitness of emergent parasitoids might be expected. Emergent parasitoids from aphids infected with *Erwinia* type bacteria were larger and more fecund than those emerging from the aphid line which harboured relatively little of either type of bacteria suggesting that cabbage aphids harbouring Group 2 *Erwinia* type bacteria are more suitable hosts for *D. rapae*. This has important implications for the ecology of cabbage aphid, since infection with Group 2 bacteria imposes both direct and indirect fitness costs to the aphids, and for our understanding of the interaction between pathogenic bacterial infection, aphids and their parasitoids. This study raises the question of why the Group 2 *Erwinia* type bacteria are

maintained over relatively long periods of time when they incur direct negative effects on cabbage aphid fitness and have a positive effect on the fitness of emergent *D. rapae*. One explanation is that the Group 2 type bacteria have another as yet undetermined fitness benefit for the aphid. For example, the secondary symbiont *R. insecticola* confers resistance to the lethal fungal pathogen *Pandora neoaphidis* on pea aphid (Scarborough *et al.* 2005) and similarly some strains of *E. herbicola* can produce antibiotics against fungal infection (Winkelmann *et al.* 1980). Since cabbage aphid is particularly vulnerable to infection with entomopathogenic fungi in laboratory culture (E. Clark *pers. obs*.), bacteria mediated resistance to fungal infection would directly benefit cabbage aphid fitness. Temporal and spatial variation in selection pressures governed by the environment (Gwynn *et al.* 2005) determine the cost or benefit of harbouring secondary bacteria at any one point in time therefore high pressure from fungal infection might be strong enough to override indirect negative effects cause by increased parasitoid fitness.

4.4.4 Additional methods would increase the validity of the results.

To date there have been very few field scale experiments investigating the dynamics of bacterial infection in natural conditions and further studies that take into account additional trophic levels are required to fully model the multitrophic nature of the field as opposed to the laboratory. In the glasshouse experiments additional parameters could be measured to increase the robustness of the results. Plant quality, which can be altered by water and nutrient stress, is known to influence aphid fecundity (Awmack and Leather 2002; Karley *et al.* 2008). Including multiple host plants in future experiments might therefore be pertinent, especially since it has been demonstrated that the parasitisation rate of *D. rapae* on cabbage aphid is influenced by the brassica type upon which the aphid is feeding (Bayhan *et al.* 2007). In the parasitoid experiments, reproductive success of parasitoids is determined by the number of individuals reaching the next generation and so counting the number of fertilised eggs might be considered as an alternative estimate of parasitoid fitness (Godfray 1994; Quicke. 1997). Unfertilised eggs do, however, have a fitness value in parasitoids in the production of virgin males, so their inclusion is warranted (Godfray 1994; Quicke. 1997). In addition, aphids have evolved a number of mechanisms to resist parasitism, which could also be recorded (Godfray 1994). At present there is no

evidence for an encapsulation response in aphids (Bensadia *et al.* 2006) similar to that observed in *Drosophila* (Fellowes and Godfray 2000). Instead, the egg breaks down quickly and fails to mature in resistant aphids (Henter and Via 1995). The lifespan of the eggs once oviposited within the aphid could be investigated using Trypan blue staining, which selectively stains dead tissues (Guay *et al.* 2009), to investigate the immune response to parasitism in cabbage aphid lines with different bacterial complements. Other measures of parasitism success include host acceptance and handling time, development time, survival and sex ratio of offspring and productivity, which is the number of new individuals emerging from a parsitised host (Godfray 1994; Antolin *et al.* 2006). Previous studies have investigated resistance to parasitism in terms of the number of aphids surviving post oviposition (Oliver *et al.* 2003; Ferrari *et al.* 2004).

By far the greatest limitation of the experiments is however the small number of aphid lines that were used. The differences in parasitism success detected in this study potentially reflect a aphid host genotype x parasitoid genotype interaction rather than bacteria mediated fitness effects alone. Clonal variation among pea aphid lines in susceptibility to parasitism suggests that there is a strong genotypic difference in this aspect of aphid fitness (Henter 1995; Henter and Via 1995). Differences in the infectivity of parasitoid genotypes have also been observed (Henry *et al.* 2008). To strengthen the results microsatellite markers could be used to establish the relatedness of the cabbage aphid lines used in this study (e.g. Caillaud *et al.* 2004). Increasing the number of cabbage aphid included in the study would also improve the validity of the conclusions (e.g. Ferrari *et al.* 2001; Ferrari *et al.* 2007). Additionally, to determine the relative importance of aphid genotype and Group 2 *Erwinia* type bacteria, cabbage aphid lines bearing Group 2 could be artificially cured using antibiotics (Koga *et al.* 2003; 2007) and their fitness compared with infected lines of the same aphid genotype. Alternatively, naturally uninfected cabbage aphid lines could be inoculated with Group 2 bacteria by microinjection (Chen and Purcell 1997). For the Group 2 *Erwinia* type bacteria and the Group 1 *Pseudomonas* type bacteria this would be relatively straightforward as these types of bacteria are amenable to in-vitro culturing (Harada and Ishikawa 1997; Grenier *et al.* 2006) and could be administered to the aphid by microinjection or even by ingestion, which might mimic the natural situation more closely. Given that variation in resistance to parasitism in pea aphids was linked to different

isolates of the symbiont *H. defensa* (Oliver *et al.* 2005), multiple isolates of each type of bacteria should be considered in future studies, along with control isolates of bacteria known to be non-pathogenic to aphids such as *E. coli* (Harada and Ishikawa 1997; Grenier *et al.* 2006).

4.4.5 Conclusions

This study has indicated there are potential fitness costs and benefits to cabbage aphids and their parasitoid *D. rapae* associated with harbouring Group 1 *Pseudomonas* type bacteria and Group 2 *Erwinia* type bacteria. The Group 2 *Erwinia* type harboured by cabbage aphid appears to have negative effects on aphid fitness and makes the aphid a more favourable host for the parasitoid *D. rapae*. Infection with Group 1 *Pseudomonas* type bacteria on the other hand appears to have a positive effect on aphid fitness and reduces the negative effects of Group 2 bacteria in double infections. However, aphid and parasitoid genotypic effects cannot be ruled out in both cases. Since only single aphid genotypes were used for this work there is a strong possibility that the results reflect differences between the fitness of aphid genotypes and are not a direct consequence of bacterial infection and therefore should be treated as preliminary. Trade-offs in aphid immunity and resistance to parasitism and bacterial pathogens are highly complex and are examined in more detail in Chapter 6. The maintenance of intraspecific biodiversity in aphid–parasitoid systems is driven by cycles of adaptation and co-adaptation between the parasitoid and its host (Sasaki and Godfray 1999; Ferrari and Godfray 2006) and the costs and benefits of harbouring bacteria in this complex interplay are only beginning to be understood. Ecological immunology distinguishes between the long-term evolutionary costs of possessing defences against natural enemies and the short term costs of using them (Sheldon and Verhulst 1996) and this study contributes to our understanding of these multitrophic interactions.

5. Molecular comparison of *Buchnera aphidicola* density in pea and cabbage aphid lines with varying secondary infection.

5.1 Introduction

5.1.1 The evolutionary origin of Buchnera *and its role in aphid ecology.*

Obligate symbioses have played a key role in shaping the diversification of many insect groups (reviewed in Janson *et al.* 2008). They have allowed a large number of herbivorous insect groups to thrive on nutrient poor plant tissues such as phloem sap and wood through provision of essential nutrients to the insect. The relationship between aphids and their primary bacterial symbiont, *Buchnera aphidicola*, is an example of an insect symbiosis that has been particularly well documented. The symbiosis between the aphid and *Buchnera* is considered 'obligate' for two reasons. Firstly in the absence of *Buchnera* development of nymphs and reproductive output of adults are both reduced (McLean and Houk 1973; Douglas 1989; Ishikawa 1989). Secondly, the genome of the bacterium has undergone a significant reduction (Charles and Ishikawa 1999; Shigenobu *et al.* 2000; Pérez-Brocal *et al.* 2006) and as a consequence *Buchnera* is viable only in its limited niche (Sasaki *et al.* 1991; Douglas 1996). The loss of genes that are essential for free living life and the maintenance of multiple genes for biosynthesis of essential amino acids to provision the host with essential nutrients (Shigenobu *et al.* 2000; Pérez-Brocal *et al.* 2006) indicates mutual dependence between the aphid and *Buchnera*. *Buchnera* are housed within primary mycetocytes and constitute greater than 90% of microbial cells in aphids and 10% of the aphid's total biomass (Douglas and Prosser 1992; Baumann and Baumann 1994). *Buchnera* is transmitted transovarially from one generation to the next (Buchner 1965; Hinde 1971). Only a small proportion of the *Buchnera* population, originating from a single maternal mycetocyte, are transmitted from the mother to the offspring (Wilkinson *et al.* 2003).

The evolutionary origin of the aphid–*Buchnera* association is ancient (160–280 million years) (Munson *et al.* 1991; Moran *et al.* 1993). *Buchnera* may have evolved from a bacterium that originally inhabited the gut of an ancestral insect host and subsequently

became incorporated into the host germ line resulting in diversification of endosymbiont and host into the present aphid species (Munson *et al.* 1991; Harada and Ishikawa 1993). Molecular phylogenies show strong evolutionary congruence and parallel diversification of *Buchnera* and its aphid hosts indicating a high fidelity of vertical transmission even at a fine scale within aphid clades (Munson *et al.* 1991; Baumann *et al.* 1995; Clark *et al.* 2000; Martinez-Torres *et al.* 2001, Jousellin *et al.* 2009). Furthermore, phylogenetic evidence based on aphid and bacterial genes suggests that *Buchnera* and aphids have undergone very strict co-speciation with no horizontal transfer of *Buchnera* even among closely related ecologically similar aphid species (Clark *et al.* 2000; Wernegreen and Moran 2001). There is extreme genome stability in *Buchnera* genomes within and between aphid species (Funk *et al.* 2000; Funk *et al.* 2001) with no gene acquisitions or chromosome rearrangements having occured in the past 50–70 million years (Tamas *et al.* 2002), indicating that genome stability of *Buchnera* has persisted through periods of aphid speciation. Consequently, given the evolutionary history of *Buchnera* spans a period including many evolutionary shifts in the diet and life cycle of its aphid host, the ecological diversity of aphids is unlikely to be explained by the genetic diversity of *Buchnera* (Funk *et al.* 2001; Tamas *et al.* 2002). Despite this apparent genetic stability, variation in *Buchnera* function has been identified and could be important in a number of aspects of aphid ecology. For example, variation in *Buchnera* function may explain differences in the production of the essential amino acid tryptophan between aphid lines (Birkle *et al.* 2002). Suppression of *Buchnera* function has been proposed as a key factor in aphid diapause (Douglas 2000) and there is evidence for both plant-mediated and natural enemy-mediated interference in the aphid–*Buchnera* symbiosis (Wilkinson *et al.* 2001; Cloutier and Douglas 2003).

5.1.2 Quantification of **Buchnera** *in aphids.*

Given the mutual dependance of aphids and their *Buchnera* symbiont, it is likely that differences in the titre of *Buchnera* could have consequences for aphid fitness. Monitoring the infection density of endosymbionts is one of the most important means of understanding their biological effects, and *Buchnera* density has been shown to vary within aphid species (Wilkinson and Douglas 1998), according to aphid morph (Hardie and Leckstein 2007), host plant type (Wilkinson *et al.* 2001) and developmental stage (Prosser

and Douglas 1992; Whitehead and Douglas 1993; Baumann and Baumann 1994; Humphreys and Douglas 1997; Mira and Moran 2002). The infection density of *Buchnera* in the pea aphid (~1 to 2 x10^7 bacterial cells per mg of adult aphid fresh weight) is reportedly the largest compared to other documented species (Prosser and Douglas 1992; Whitehead and Douglas 1993; Humphreys and Douglas 1997; Wilkinson and Douglas 1998). Smaller aphid species such as *Schizaphis graminum* and *Aphis fabae* harbour fewer *Buchnera* cells than larger aphids (Baumann and Baumann 1994; Wilkinson *et al.* 2001) indicating that *Buchnera* infection is a function of aphid size as larger aphids are likely to harbour more mycetocytes. The pea aphid (*A. pisum*) has been the main focus of studies to document *Buchnera* infection using a variety of methods including quantitative hybridization, microscopy and quantitative PCR (Prosser and Douglas 1992; Whitehead and Douglas 1993; Humphreys and Douglas 1997; Wilkinson and Douglas 1998). Another small aphid species was investigated in this study, the cabbage aphid (*Brevicoryne brassicae*). A. *pisum* and *B. brassicae* are members of different sub-tribes (Macrosiphina and Liosomaphidina respectively), and feed on an entirely different family of host plants. *B. brassicae* is an obligate feeder on crucifers and a common pest species of brassica crops throughout the United Kingdom while *A. pisum* feeds on legumes and is a minor pest in the UK.

5.1.3 The influence of secondary symbiont infection on Buchnera *density.*

In addition to *Buchnera*, *A. pisum* is known to harbour several secondary symbiont bacteria which have been shown to affect the dynamics of the aphid–*Buchnera* symbiosis (Fukatsu *et al.* 2000; Darby *et al.* 2001; Sandström *et al.* 2001; Haynes *et al.* 2003). In particular, the secondary symbiont *Serratia symbiotica* can have a marked impact on *A. pisum–Buchnera* responses to diet and plant type (Wilkinson *et al.* 2001; Wilkinson *et al.* 2007). Pea aphids harbouring *S. symbiotica* or *Rickettsia* symbionts frequently show reduced densities of *Buchnera* (Koga *et al.* 2003; Sakurai *et al.* 2005; Koga *et al.* 2007). In this study, *B. brassicae* does not harbour the secondary symbiont, *S. symbiotica*. Instead *Pseudomonas* and *Erwina spp.* are present (Chapter 3) and these bacteria might influence the density of *Buchnera* in *B. brassicae*. To address this possibility, quantitative real-time Taqman® PCR was used to determine the relative infection density of *Buchnera* in nine *B.*

brassicae lines and nine *A. pisum* lines with known secondary symbiont infection status. The density of *Buchnera* was compared between aphid lines that either harboured secondary bacteria or had no secondary infection. *Buchnera* specific sequence for the single copy gene *GroEL* was used to quantify *Buchnera* in each aphid line relative to aphid reference genes elongation factor 1-α (*Ef1-α*) and β-tubulin. The aim of this experiment was to determine whether two aphid species differed consistently in their density of *Buchnera*, and whether secondary bacteria had an impact on the titre of *Buchnera.*

5.2 Materials and Methods

5.2.1 Experimental aphids.

A subset of nine of the original cabbage aphid *B. brassicae* clonal lines, collected from brassica plants in field sites in Fife and Tayside (Table 5.1), were used to investigate variation in the density of *Buchnera* within and between different cabbage aphid lines. In addition to the cabbage aphid lines, several pea aphid (*A. pisum*) lines were used in the study (Table 5.2). The pea aphids were provided as established clonal lines from laboratory cultures by Professor Angela Douglas (University of York) and Dr Glen Powell (Imperial College). All aphids were reared in culture conditions described previously (Section 2.2.1) and pea aphids were maintained on broad bean *Vicia faba* cultivar The Sutton.

The secondary symbiont status of the pea aphid lines was determined by Hannah Clarke using the diagnostic PCR assay described in Section 2.2.4.1, which was also previously used (Section 2.3.2) to verify that the three secondary symbionts, *Hamiltonella defensa*, *Serratia symbiotica* and *Regiella insecticola* were absent from all the cabbage aphid lines.

Table 5.1: Code, collection date, location and plant type for experimental cabbage aphid populations.

Code	Date Collected	Location	Collection Plant
ELC0604	11/10/2006	Tayport, Fife	Cabbage
ELC0605	12/10/2006	Dundee. Tayside	Brussels Sprout
ELC0606	12/10/2006	Letham, Fife	Broccoli
ELC0607	12/10/2006	Tayport, Fife	Purple Sprouting Broccoli
ELC0610	13/10/2006	Balmullo, Fife	Kale
ELC0611	17/10/2006	Dundee, Tayside	Cabbage
ELC0619	25/10/2006	Dundee, Tayside	Turnip
ELC0701	10/07/2007	Invergowrie, Tayside	Oil Seed Rape
ELC0703	20/08/2007	Tayport, Fife	Brussels Sprout

Table 5.2: Code, provider, original collection plant and symbiont infection status for pea aphid lines. ***In other published work (e.g. Douglas *et al.* 2006a) TLW03/01 has been shown to harbour *S. symbiotica* but this particular line was shown by diagnostic PCR not to carry the infection.**

Code	Provided by	Collection Plant	Symbiont Status
TLW03/01	Angela Douglas	*Medicago sativa*	None*
JF99/04	Angela Douglas	*Lotus pedunculatus*	*H. defensa*
JF98/24	Angela Douglas	*Vicia faba*	*R. insecticola*
PS01	Glen Powell	*Vicia faba*	*S. symbiotica*
LL01	Glen Powell	*Medicago sativa*	None
N127	Glen Powell	*Medicago sativa*	*H. defensa*
N116	Glen Powell	*Medicago sativa*	*H. defensa*
JF01/29	Glen Powell	*Lathyrus pratensis*	*S. symbiotica*
SH3	Glen Powell	*Pisum sativum*	*S. symbiotica*

5.2.2 DNA extraction from single aphids.

Single adult cabbage and pea aphids of approximately equal size for each species were selected prior to their first larviposition and stored at -20°C. DNA was extracted using a DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, California). The extraction protocol was modified for efficiency of use on single aphid samples, to include an additional 10 minute lysis step and a smaller elution volume, following the Qiagen Supplementary Protocol: Purification of total DNA from insects using the DNeasy® Blood & Tissue Kit (DY14-Aug06). Initially, single aphids were macerated in a 2 ml Eppendorf using an ethanol-sterilised micropestle while suspended in liquid nitrogen. Once aphid material was thoroughly broken down 180 µl of buffer ATL was added with 20 µl of Proteinase K and the solution was vortexed. Samples were then incubated at 55ºC on a heat block (Grant QBTP Heat Block, Grant Instruments, UK) for one hour and vortexed every fifteen minutes. After an hour the samples were vortexed for fifteen seconds and 200 µl of buffer AL was added before the samples were vortexed again and incubated at 70ºC on a heat block for ten minutes. After ten minutes 200 µl of 100% ethanol was added to the samples and they were vortexed thoroughly. Samples were then transferred onto the centre of the membrane of a DNeasy mini spin column placed in 2 ml collection tubes and then centrifuged at 6000 *g* (Eppendorf Desktop MicroCentrifuge 5424, Eppendorf, UK) for one minute. Next, the columns were washed twice, first with 500 µl of buffer AW1 and centrifuged for one minute at 6000 *g*, then with 500 µl of buffer AW2 and centrifuged for three minutes at 18,400 *g* using a new collection tube each time. After washing the spin columns were placed in a sterile 1.5 ml Eppendorf and 100 µl of buffer AE was added to the centre of each spin column membrane. The columns were then incubated at room temperature for one minute and centrifuged for one minute at 6000 *g* to elute the DNA and complete the kit protocol.

The concentration of the eluted DNA was measured on a Full Spectrum UV/Vis NANODrop Spectrophotometer (ND-1000, Labtech International). Concentrations greater than 5 ng/µl were considered sufficient for quantitative PCR. Eluted DNA was then divided into 20 µl aliquots to prevent freeze-thaw degradation of the samples and stored at -20ºC.

5.2.3 Real-time (SYBR®) quantitative PCR assay development for **Buchnera** *quantification.*

5.2.3.1 SYBR® Green I assay design.

The real-time PCR assays incorporated SYBR® Green I technology with specific forward and reverse primers for the *Buchnera GroEL* gene in cabbage and pea aphid. Real-time PCR primers were designed to amplify a short amplicon (~80 bp) of the target nucleotide sequence from the *Buchnera GroEL* gene in pea and cabbage aphids. Initially, the previously published primers Buch*GroEL*-1824F (5'-CGTTTCAGATCCATTGGAT TCA-3') and reverse primer Buch*GroEL*-1967R (5'-AGCTCAAATGGTAAAAGAAGTTGCA-3') (Genbank accession number X61150) (Sakurai *et al.* 2005) were used to quantify *Buchnera GroEL* but melt curve analysis suggested that these primers did not completely match the target template. Melt curve analysis following the qPCR reaction monitors the dissociation characteristics of double stranded DNA during heating for sequence verification (Wittwer *et al.* 2003). When the fluorescence level of the SYBR® Green dye, which is a DNA-intercalating fluorophore, is plotted against temperature, differences in peak number, peak position or a combination of both can occur in the melt curves of two samples indicating nucleotide polymorphisms between amplicons (Wittwer *et al.* 2003). The peak position varied for the *Buchnera GroEL* amplicons generated for pea and cabbage aphid indicating sequence variation between the two species, which could influence qPCR amplification efficiency. Subsequent cloning and sequencing (Section 2.2.5.1) of the *GroEL* product revealed differences in the sequence at the primer sites in cabbage and pea aphid compared to those published for pea aphid. Consequently, the primers were redesigned from *GroEL* sequence generated using the original Sakurai *et al.* (2005) primers from two cabbage and two pea aphid lines. Sequences were aligned in POA and the alignment was viewed using Genedoc (http://www.psc.edu/biomed/genedoc) to determine suitable primer sites for both aphid species (Fig. 5.1). Two sets of primers were manually designed: pea aphid forward APBuchGroELF (3'-GCTACTGCTTTTAAACCTTCATTTAC-5') and pea aphid reverse APBuchGroELR (5'-CAGCAGGTGATGGTACCACAA-3') and cabbage aphid forward BBBuchGroELF (5'-CTACTGCTTTTAGACCTTCGTTTACT-3') and cabbage aphid

reverse BBBuch*GroEL*R (5'-CAGCAGGAGATGGAACAACAA-3'). Both sets of primers were checked for efficiency using Netprimer software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) and verified for specificity by a Basic Local Alignment Search Tool (BLAST) search on the NCBI sequence database.

Two aphid nuclear genes, aphid elongation factor 1α (*Ef*1-α) and β-tubulin were chosen as suitable endogenous reference genes to standardise for the amount of aphid DNA added to the reaction. *Ef1*-α was quantified using primers ApisEF1-422F (5'-CTCTGGATGGAATGGAGACAACA-3') and ApisEF1-522R (5'-ATTTACCGTCGGCCTTTCCT-3') (Sakurai *et al.* 2005) and β-tubulin was quantified using primers βTubF (5'-GGCCAAGGGTCATTACACTGA-3') and βTubR (5'-TGCGAACCACGTCCAACA-3') (Accession number: APD01867) (Shakesby *et al.* 2009).

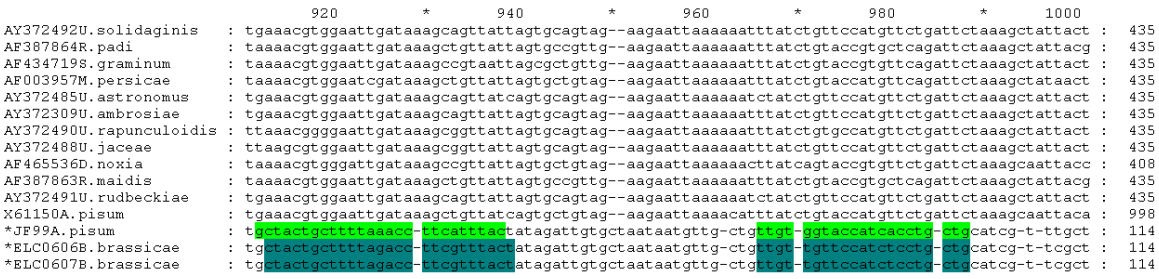


Figure 5.1: **Buchnera GroEL** ***sequence from multiple aphid species aligned to illustrate the positions of the APBuchGroELF and APBuchGroELR primers (light green) for pea aphid and the BBBuchGroELF and BBBuchGroELR primers (dark green) for cabbage aphid. NCBI Accession numbers precede each sequence with the exception of those generated for this study which are preceded by *.***

5.2.3.2 General real-time PCR conditions.

Real-time PCR (SYBR®) reactions were set up in 96-well reaction plates (Eurogentec, Belgium) and cycling and data collection were performed using an ABI 7500 FAST Real-time PCR System (Applied Biosystems, Warrington, UK). For each reaction 1 µl of DNA template was added to 11 µl of reaction mix in the appropriate well. Primer concentration matrices were used to optimise the concentration of primer used in a 200, 400 and 800 nM 3 x 3 set up (User Bulletin 2, Applied Biosystems) and reaction efficiency was compared over a four point dilution series at each matrix concentration combination. The primer combination that gave the lowest (Ct) value was chosen as the optimal concentration for all reactions. In each case the optimal concentration was 400 nM for both primers. A MESA Blue qPCR MasterMix Plus for SYBR Assay Low Rox kit (Eurogentec, Belgium) was used for all qPCR SYBR® reactions. The PCR reaction mixture contained 1x PCR reaction buffer (containing dNTPs, Meteor*Taq* DNA polymerase, 4 mM $MgCl_2$, SYBR® Green I, blue dye, ROX passive reference and stabilisers), 400 nM each primer and 1 µl DNA extract template in a 25 µl reaction. The reaction conditions were 95ºC for 5 minutes to melt the DNA, followed by 45 cycles of 95ºC for 3 seconds to anneal the primers and an elongation step of 60ºC for 1 minute followed by a dissociation stage of 95ºC for 15 seconds, 60ºC for 1 minute and 95ºC for 15 seconds to generate the melt curves. No template control (NTC) reactions, which contained 1 µl of water instead of DNA, were included for each primer pair to identify any contamination of reactions. Melt curve analysis was used to determine the specificity of the reaction according to the consistency of the peak position on the melt curve when DNA template was added and the lack of any peak in the no template controls. The presence of primer–dimer in all reactions was also determined according to the dissociation characteristics of the double-stranded DNA. Primer–dimers are aberrant PCR products that are formed due to complementarity between the primers, particularly in the 3'-end (Kubista *et al.* 2006). Competition between PCR of the target and PCR forming primer–dimers compete therefore avoiding the formation of primer–dimer products is very important for quantitative PCR analysis (Kubista *et al.* 2006). If primer–dimers were present multiple peaks were observed in the melt curves. Any inconsistency occurring in the melt curve analysis meant reactions were repeated.

5.2.3.3 Primer validation experiments.

Validation experiments were performed by comparing the amplification efficiency of the target gene and the two reference genes in a 100-fold dilution series (1/10 to 1/1000 of aphid DNA ≥5 ng/µl in each dilution) in triplicate of total DNA extracted from single pea and cabbage aphids. The reference gene primers were tested with both pea and cabbage aphid DNA while the *GroEL* primers designed specifically for each aphid species were tested on DNA from the corresponding aphid species. The Ct values were plotted against dilution level to produce standard curves for each assay (Fig. 5.2 A to F). In an ideal reaction the Ct values would decrease as the dilution level increased. Optimal criteria for efficiency of the PCR reactions were an absolute value of the slope of Ct versus dilution of >-3.1 and a correlation coefficient R^2 value of >0.985 (User Bulletin 2, Applied Biosystems). All standard curves indicated the primers fit these criteria at a 400/400 nM final concentration of each primer per reaction (Fig. 5.2).

5.2.3.4 Relative quantification using the comparative Ct method.

The comparative cycle threshold (Ct) method of relative quantification described previously (Chapter 3) was chosen as the most suitable method of quantification for this study. To investigate the effect of secondary infection on *Buchnera* density, an aphid line of the same species with no detectable secondary infection was used as a calibrator while differences in *Buchnera* density between the two aphid species were compared using an aphid line from the opposite species as a calibrator. In order to use the ΔΔCt method it was essential that the amplification efficiency of the endogenous and target genes was approximately equal i.e. the *GroEL* gene and the two reference genes amplified equally well at low and high DNA concentrations for both aphid species. A 100-fold dilution series (1/10 to 1/1000 of ≥5 ng/µl aphid DNA) of aphid DNA from a single pea or cabbage aphid, assayed in triplicate was used to determine the amplification efficiency of the pea and cabbage aphid assays. In each case the slope of a fitted regression line was either <0.1 or between 0.1 and 0.15, and the linear correlation coefficient (r) values were close to 0, thus the primer amplification efficiencies of the target and endogenous genes were considered equal and suitable for using the comparative Ct method (Fig. 5.3).

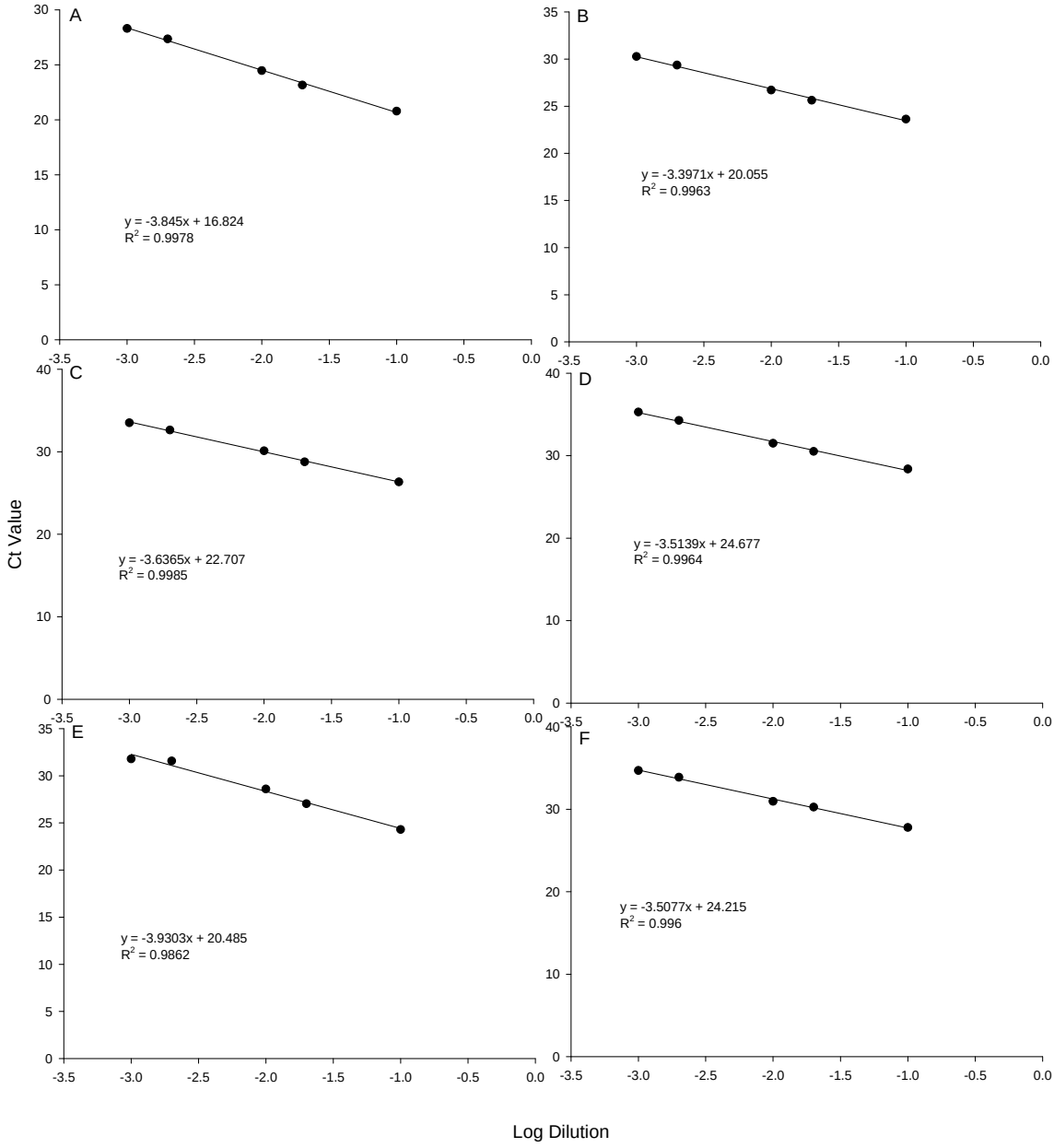


Figure 5.2: Standard curves for detection of (A and B)* Buchnera GroEL, *(C and D)* Ef1-α *and (E and F) β-tubulin in (A, C and E) pea aphid and (B, D and F) cabbage aphid using SYBR® assays.

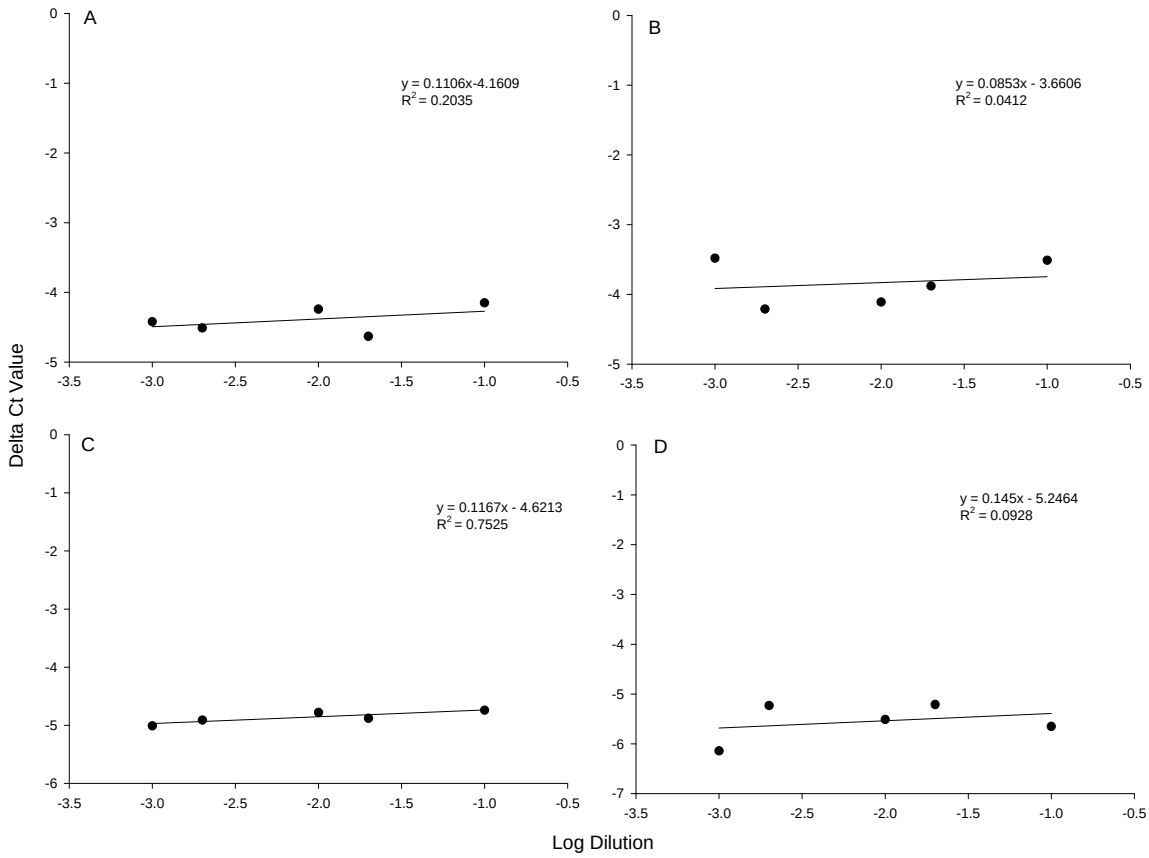


Figure 5.3: A plot of ΔCt (target-reference) to assess the primer amplification efficiency for the target* (GroEL) *and reference (A and B) β-tubulin gene and (B and C)* Ef1-α *gene for (A and C) cabbage aphid and (B and D) pea aphid.

To ensure there was a correlation between the results obtained for the two reference genes, linear correlation was performed on the Ct values in Genstat (Version 10.2). If the two genes were in agreement there would be a linear relationship between the Ct values. Analysis showed a strong positive correlation between the Ct values for both reference genes for pea aphid, Linear Regression, df = 107, r = 0.805, p <0.001 and for cabbage aphid, Linear regression, df = 97, r = 0.751, p <0.001. The strong correlation between the two reference genes supported their suitability as reference genes for estimating aphid DNA content in relative quantification (Fig. 5.4). Overall the Ct values were slightly higher for β-tubulin in cabbage aphid than *Ef1-α* suggesting that the primer efficiency for β-tubulin was slightly reduced with cabbage aphid tissue. This was not entirely unexpected as the primers were designed by Shakesby *et al.* (2009) for pea aphid and therefore may be less well matched to cabbage aphid sequence than the *Ef1-α* primers.

5.2.3.5 Application of the SYBR® assay and analysis of results.

Once optimised the SYBR® assay was used to determine the number of *Buchnera* genome copies per aphid reference gene in six individual adult aphids from nine different pea and cabbage aphid lines. A volume of 1 µl of DNA template from each aphid sample (diluted prior to adding to the reaction 1/25 for initial concentrations of neat aphid DNA of <100 ng/µl and 1/50 for concentrations >100 ng/µl) was added to each realtime PCR reaction and all reactions were duplicated for accuracy. The infection density of *Buchnera* in this study equals the number of *Buchnera* genes per aphid gene thus assuming a relationship between the number of genes and the number of cells and therefore the size and weight of the aphids. Pre-reproductive adult aphids of similar size were selected to limit the effects of ploidy and developmental stage on the results. As detailed previously the comparative Ct method was used to determine the relative density of the *Buchnera GroEL* gene in the different aphid lines relative to the density of *Buchnera GroEL* in a calibrator aphid line. The calibrator aphid line was either of the opposite aphid species or with no other secondary symbiont infection (pea aphid), or with relatively little of either the *Pseudomonas* (Group 1) or *Erwinia* (Group 2) groups of accessory bacteria (cabbage

aphid). Statistical analysis of the ΔCt values using ANOVA was performed in Genstat (Version 10.2) to determine the impact on *Buchnera* density of aphid species and of secondary bacteria status. Two reference genes were included to increase confidence in the results in line with recent research that emphasises the importance of using multiple reference genes in qPCR analysis (Vandesompele *et al.* 2002).

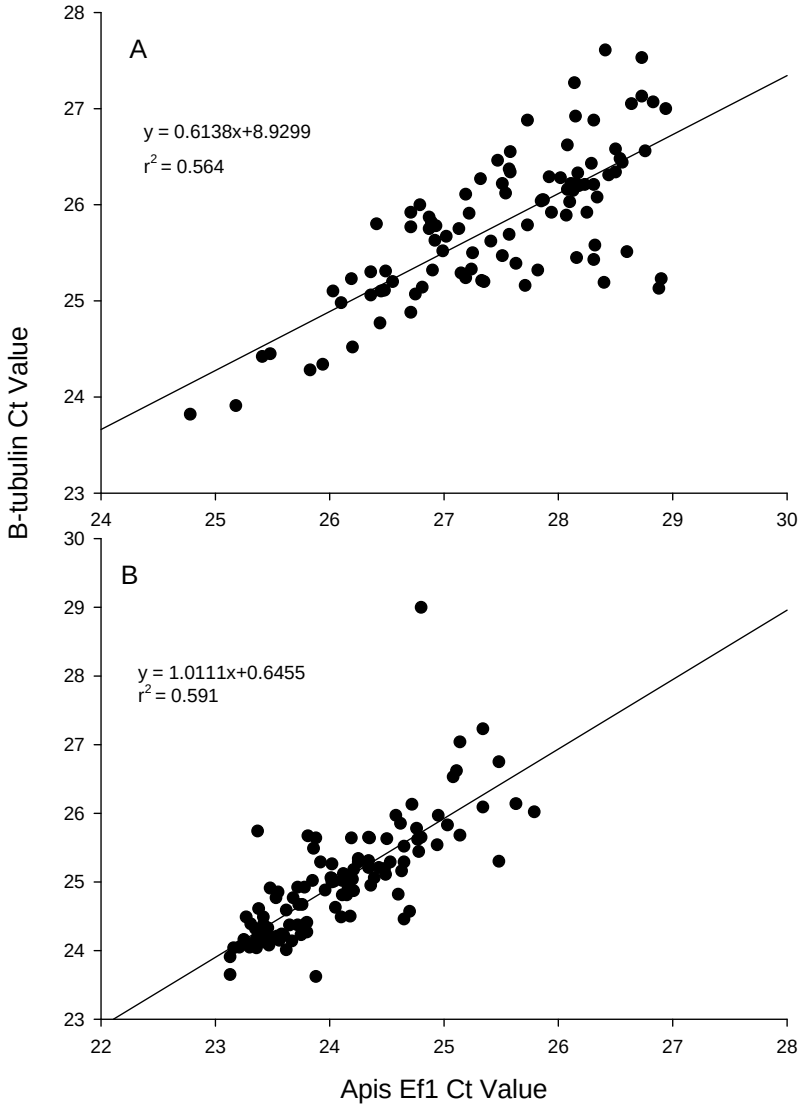


Figure 5.4: Correlation between Ct values for the two endogenous reference genes* Ef1-α *and β-tubulin in (A) cabbage aphid (N = 88) and (B) pea aphid (N = 108).

5.3 Results

5.3.1 Quantification and comparison of Buchnera GroEL *in two aphid species.*

Initial comparison of *Buchnera GroEL* in the two aphid species was based on the relative quantities for each aphid line calculated using the comparative Ct method detailed previously (Section 3.2.3.5) (Applied Biosystems User Bulletin 2). In both aphid species, a calibrator aphid line from the other species was chosen which had relatively little of either Group 1 or Group 2 type bacteria (cabbage aphid line ELC0610), or no infection with any characterised secondary symbiont type (pea aphid line LL01). Using aphid lines free from secondary bacteria as calibrators would allow quantification of any effects of secondary infection on the density of the primary symbiont in the two aphid species.

The relative quantity of *Buchnera GroEL* was greater in the cabbage aphid than the pea aphid, which was contrary to what was expected, since the pea aphids used in this study were three times larger than the cabbage aphids. As an estimation of the difference in size between the two aphid species, the average weight of a sample of 10 pea and cabbage aphids selected randomly from the clonal lines was calculated and cabbage aphids were found to be significantly smaller (One way analysis of variance $F_{1,20} = 120.08$, $p < 0.001$; cabbage aphid mean = 0.7985+/-0.0583 mg and pea aphid mean = 3.088+/-0.3842 mg (+/- 95% confidence interval)) . In each pea aphid line the relative density of *Buchnera GroEL* was less than in the cabbage aphid calibrator (Fig 5.5; Appendix 4.1). Pea aphid line PS01 exhibited the greatest difference with three fold lower densities of *Buchnera* than ELC0610 the cabbage aphid line, while TLW03/01 and LL01 exhibited smaller differences compared to the cabbage aphid line. This might relate to the fact that neither TLW03/01 nor LL01 harboured any secondary symbionts while PS01 was positive for *S. symbiotica* indicating a potential effect of secondary infection on *Buchnera* density. Most cabbage aphid lines had relative densities of *Buchnera* that were approximately 1.5 fold higher than the pea aphid calibrator (LL01), with the exception of line ELC0703 which had a smaller relative density of *Buchnera* than LL01 (Appendix 4.2). The two reference genes gave similar clonal patterns in relative quantity of *Buchnera GroEL* (Fig 5.5; Appendix 4.1 & 4.2).

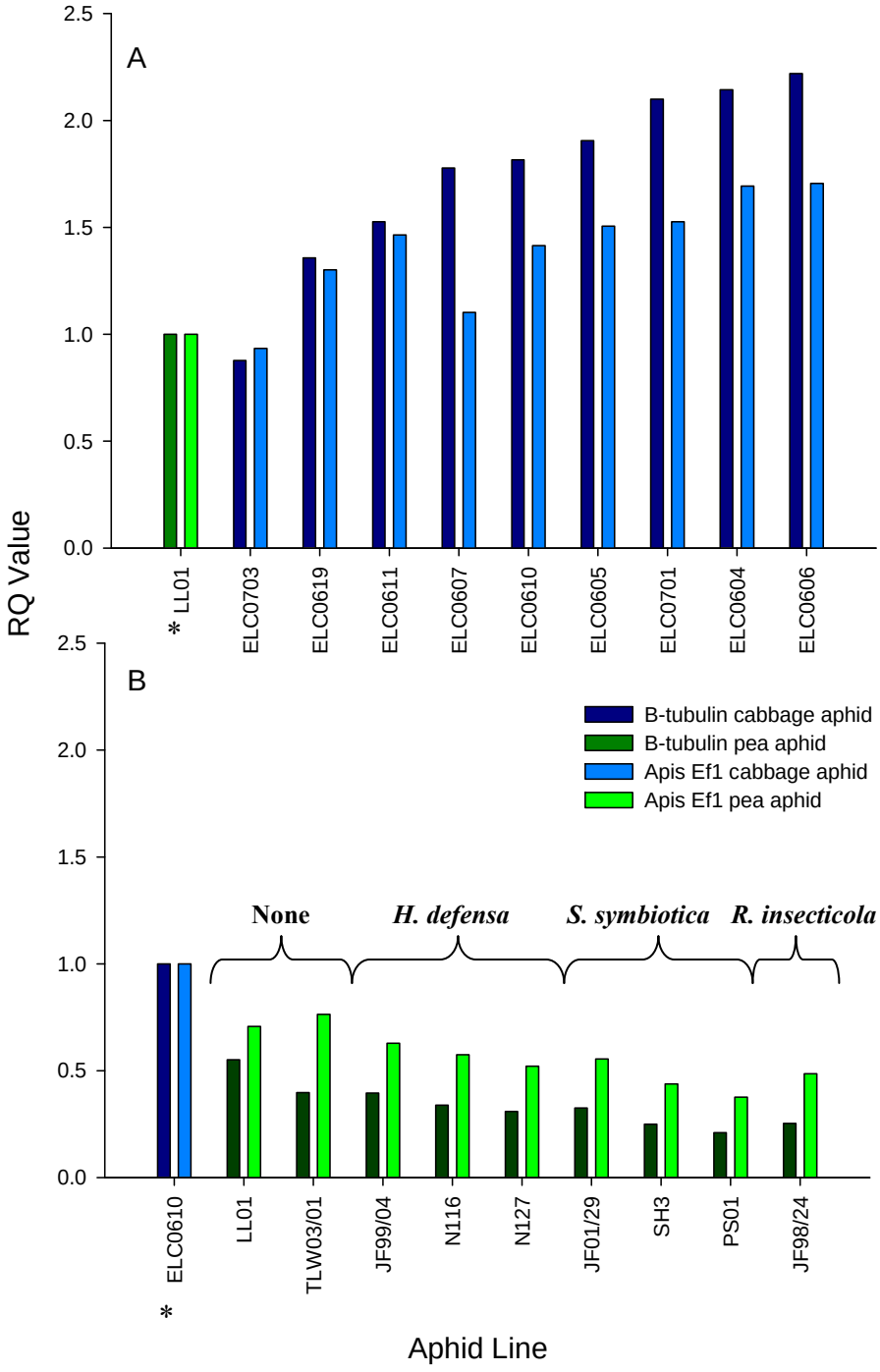


Figure 5.5: Relative quantification of* Buchnera GroEL *in (A) cabbage aphid and (B) pea aphid relative to a calibrator aphid line with no secondary infection respectively, pea aphid (LL01) and cabbage aphid (ELC0610) (calibrator shown in a different colour and denoted by *).

Significantly higher relative quantity of *Buchnera* in cabbage aphids compared to pea aphids was confirmed using both reference genes *Ef1-α* and β-tubulin (One-way analysis of variance $F_{1,210} = 55.53$; $p < 0.001$ and $F_{1,207} = 161.86$; $p < 0.001$ respectively) which was reflected in the relative quantity (RQ) calculated for each aphid line (Fig. 5.5; Appendix 4.1 & 4.2). A higher relative quantity of *GroEL*, which is a single copy gene in the *Buchnera* genome, is likely to reflect higher densities of bacterial cells in cabbage aphids compared to pea aphids, although the possibility that polyploidy in the *Buchnera* genome contributed to within and between aphid species differences cannot be ruled out.

5.3.2 Quantification and comparison of **Buchnera GroEL** *within pea aphid lines with varying secondary symbiont infection status*.

Relative quantification of the *Buchnera GroEL* gene revealed differences across the pea aphid lines (Appendix 5.1; Fig. 5.6). The rank order of the aphid lines from highest (1) to lowest (9) relative densities of *Buchnera* were identical for the two reference genes, increasing confidence in their suitability as endogenous references (Table 5.3). However, when the differences in *Buchnera GroEL* relative to *Ef1-α* and β-tubulin were further investigated by statistical analysis of the ΔCt values, the two reference genes yielded different outcomes. The quantity of *Buchnera GroEL* gene did not vary significantly between different pea aphid lines when *Ef1-α* was used as the reference gene (One-way analysis of variance $F_{8,106} = 1.5$; $p = 0.166$) but there was a significant difference when β-tubulin was used as the reference gene (One-way analysis of variance $F_{8,106} = 2.41$; $p = 0.020$). The confounding result for the two different reference genes is odd particularly given that both show similar orders of magnitude difference in relative quantification values. The *Ef1-α* reference genes did however exhibit larger variation within aphid lines, which could at least partially account for the result.

The extent to which secondary symbiont infection status affected the difference in the relative quantity of the *Buchnera GroEL* gene across the aphid lines was difficult to determine fully as *S. symbiotica*, *H. defensa* and *R. insecticola* were not quantified in this study. There was a significant effect of secondary symbiont infection status on the relative quantity of *Buchnera* across the pea aphid lines using *Ef1-α* as a reference (One-way analysis of variance $F_{3,8} = 6.95$, $p = 0.031$) but the effect was weaker when β-tubulin was

used as the reference gene (One-way analysis of variance $F_{3,8} = 4.65$, $p = 0.066$). In both cases two of the pea aphid lines harbouring *S. symbiotica* (PS01 and SH3) contained the lowest densities of *Buchnera GroEL* relative to line LL01 (Table 5.3), which was negative for the three secondary symbiont types tested in this study. Consequently, the results suggested that in the presence of *S. symbiotica*, *Buchnera* infection density was reduced. In addition, the aphid line that contained the greatest quantity of bacteria relative to LL01 was another aphid line that harboured none of the three secondary symbiont types, TLW03/01, indicating that the higher densities of *Buchnera* are maintained in the absence of secondary symbionts (Table 5.3).

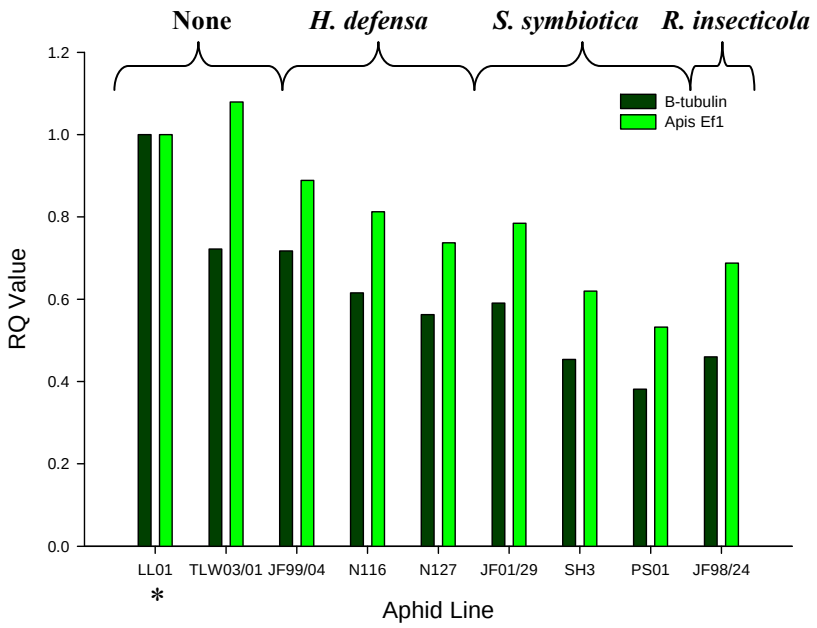


Figure 5.6: Relative quantification of* Buchnera *GroEL (RQ value) in pea aphid lines using LL01 which had no secondary infection as a calibrator (denoted by *).

Table 5.3: Secondary infection status of pea aphid lines and corresponding RQ values ranked from most (1) to least (9) for relative copy number of the* Buchnera GroEL *gene.

Aphid Line	Secondary Infection	Relative Quantity of *Buchnera* (RQ) β-tubulin	*Ef1-α*	Rank
LL01 Calibrator	None	1	1	1
TLW03/01	None	0.72	1.08	2
JF99/04	*H. defensa*	0.72	0.89	3
N116	*H. defensa*	0.62	0.81	4
N127	*H. defensa*	0.56	0.74	6
JF01/29	*S. symbiotica*	0.59	0.79	5
SH3	*S. symbiotica*	0.45	0.62	8
PS01	*S. symbiotica*	0.38	0.53	9
JF98/24	*R. insecticola*	0.46	0.69	7

5.3.3 Quantification and comparison of **Buchnera GroEL** *within cabbage aphid lines with varying secondary bacterial infection status*.

Relative quantification calculations (Fig. 5.7A; Appendix 5.2) and statistical comparison of the ΔCt values revealed that the density of *Buchnera GroEL* gene varied significantly between cabbage aphid lines (One-way analysis of variance $F_{8,104} = 2.38$, $p = 0.022$ and $F_{8,101} = 6.44$, $p < 0.001$ for *Ef1-*α and β-tubulin respectively). When differences in the relative copy number of the two dominant groups of secondary bacteria (Group 1 – *Pseudomonas* and Group 2 – *Erwinia*) (Fig. 5.7B; Appendix 5.2) across the cabbage aphid lines were compared with the relative quantity of *Buchnera GroEL* there was no clear relationship between *Buchnera* density and secondary infection status (Fig. 5.7A&B; Appendix 5.2). In aphid line ELC0703 relatively low densities of *Buchnera GroEL* were associated with relatively low densities of Group 1 and 2 secondary bacteria. However, low densities of Group 1 and 2 bacteria were associated with high densities of *Buchnera* in aphid line ELC0701 indicating that there was no consistent relation between primary and secondary bacteria densities. There was also no evidence that dominance of one or both groups of accessory bacteria was related to the relative density of *Buchnera GroEL* between cabbage aphid lines. Consequently, the accessory bacteria are unlikely to be the cause of variation in the density of *Buchnera* in the cabbage aphid lines.

Two-way analysis of variance was used to test the effect of the additional explanatory variables 'real-time replicate run' and 'individual aphid' with the original explanatory variable 'aphid line' on the variability in the data. Each real-time reaction was duplicated for accuracy, thus replicate run would have an effect on the variability in the data if reactions were inconsistent due to pipetting errors, for example. There was no significant effect of real-time replicate run on the response variable indicating a high level of consistency in the real-time reactions. However, the effect of individual aphid was significant in each case suggesting the *Buchnera* ploidy within individual aphids was variable and may account for some of the observed variation, or that *Buchnera* density is very variable between individual aphids.

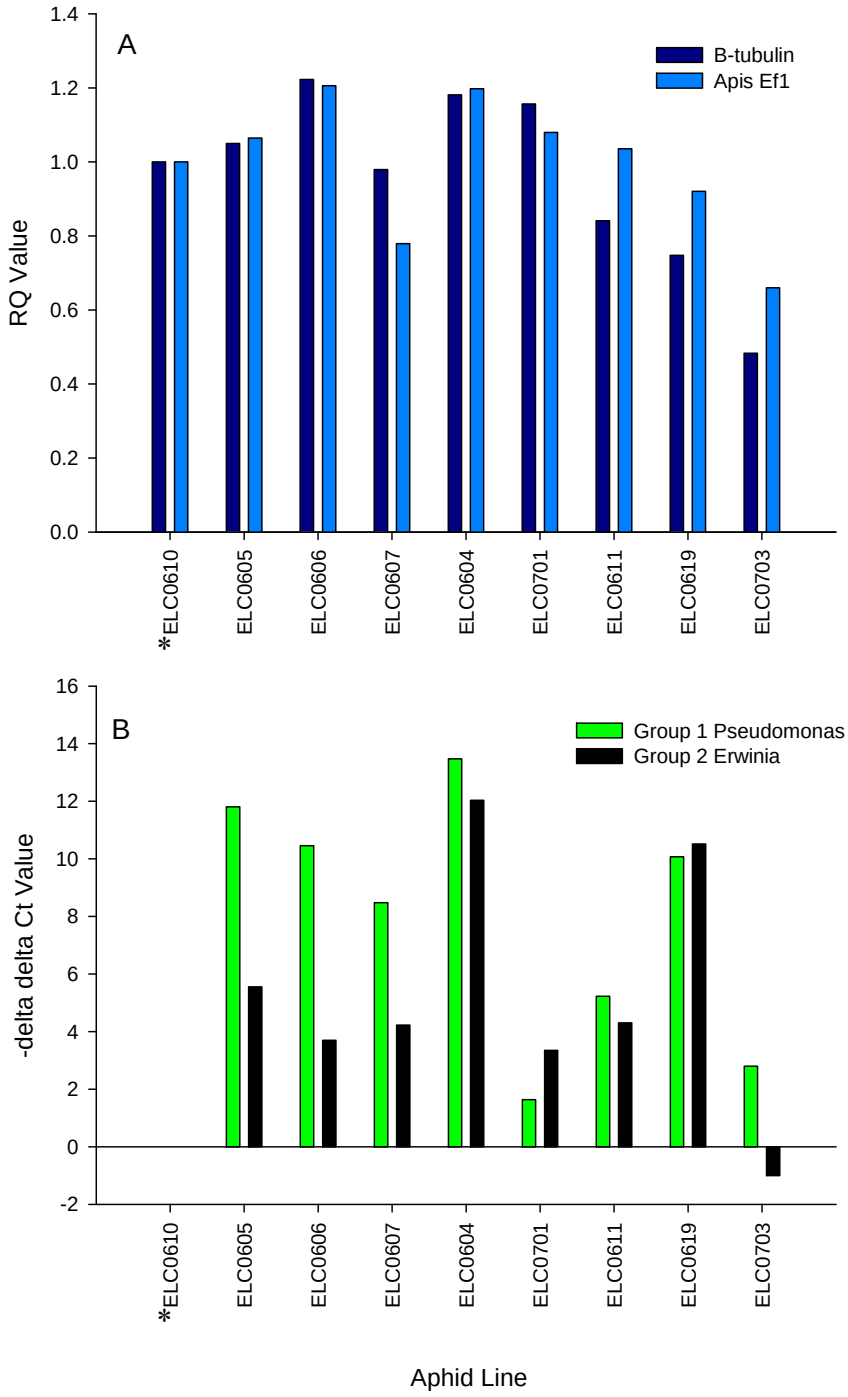


Figure 5.7: Comparison of (A)* Buchnera GroEL *density and (B) differences in the relative copy number of the 16S gene for the Group 1 and 2 bacteria across cabbage aphid lines. (Both were quantified relative to the calibrator line ELC0610 denoted by *)

5.4 Discussion

5.4.1 Cabbage aphid harboured higher relative densities of Buchnera *than pea aphid.*

Although the nature and function of the *Buchnera* symbiosis in insect physiology and evolution is well understood, we know relatively little either about how the symbiosis varies between different aphid species, or the effects of secondary bacteria. This study demonstrated clearly that the relative abundance of *Buchnera* varied quantitatively between and within two different aphid species. Factors underlying this variation could include the effects of initial collection plant (Wilkinson *et al.* 2001; Wilkinson *et al.* 2007) and other ecological variables such as temperature (Baumann *et al.* 1996) or infection with other bacteria (Koga *et al.* 2003; Sakurai *et al.* 2005; Koga *et al.* 2007). In particular, the variation within the pea aphid lines might relate to presence and type of secondary symbiont. *S. symbiotica* is known to depress *Buchnera* infection density in pea aphids possibly as a result of competition for resources (Koga *et al.* 2003; Sakurai *et al.* 2005; Koga *et al.* 2007) and a similar trend was observed in this study for two of the three pea aphid lines harbouring this secondary symbiont. The factors underlying variation in *Buchnera* density across the cabbage aphid lines were less clear and did not relate to the presence of relative abundance of secondary bacteria.

Secondary symbiont infection could not explain the differences between aphid species in *Buchnera* density. Cabbage aphid lines harboured significantly higher densities of *Buchnera* than pea aphid lines, even when aphid lines with no secondary bacteria were compared. A previous study by Baumann and Baumann (1994), using a qPCR method based on the *Buchnera rrs* gene, found that *S. graminum*, the smaller of the two aphid species, had about 30 times less *Buchnera rrs* genes than *A. pisum* relative to the number of aphid genes. The opposite effect on *Buchnera* density was recorded in the cabbage aphid, which is also smaller than the pea aphid. The ecological significance of this is unclear. It could be speculated that cabbage aphid harbours more *Buchnera* to compensate for either the energetic requirments of posessing secondary defences or for feeding on host plants with lower nutritional value. However, the cabbage aphid–*Buchnera* symbiosis has not been characterised using the genomic and transcriptomic tools that have been applied to the pea aphid (e.g. Thomas *et al.* 2009). Application of molecular approaches, combined with

microscopy studies could confirm whether higher densities of *Buchnera* in the cabbage aphid relate to differences in mycetocyte number and genetic control of *Buchnera* function.

5.4.2 Aphid developmental stage and physiology may also be key factors influencing **Buchnera** *density.*

A further explanation for variation in the *Buchnera* density across aphid lines relates to aphid developmental differences in *Buchnera* infection. An increase in the genomic copy number of *Buchnera* during post–embryonic development to adulthood followed by a decrease as the host aged has been reported for the pea aphid (Komaki and Ishikawa 1999; 2000). Similarly, *Buchnera GroEL* density rose following an increase in aphid weight with an average of $1.6x10^5$ molecules of *Buchnera GroEL* per μm^3 cell volume (Baumann *et al.* 1997). The actual number of endosymbionts per aphid was reported to increase from $0.2x10^6$ at birth to $5x10^6$ endosymbionts at maximum weight (10–11 days) (Baumann and Baumann 1994). To minimise the effect of insect developmental stage on the results all the aphids used for this study were adult aphids selected prior to their first larviposition. It is possible that small differences in aphid developmental stage, particularly in the time between reaching adulthood and initiation of larviposition (approximately 0–3 days), could explain some of the variation in the data. It is also important to note that conclusions relating to relative density of *Buchnera* at the stage in aphid development investigated in this study may differ for younger or older aphids.

Variation in *Buchnera* density could also be explained by the effects of the reference genes used. Evidence suggests that the relative levels expression of some reference genes can vary across animal tissues (Thellin *et al.* 1999) though since DNA, rather than RNA, was used in this study that is not an essential consideration. Aphids reproduce by parthenogenesis with the formation of embryos within embryos, thus necessitating consideration of the contribution of embryonic tissue and *Buchnera* within embryos in the qPCR analysis. A small proportion of *Buchnera* (~800 symbionts) are transmitted transovarially to the embryo via a stream of bacteria from a single mycetocyte in the adult at stage 7 of embryo development immediately before anatrepsis (Mira and Moran 2002; Miura *et al.* 2003; Wilkinson *et al.* 2003). Consequently, any quantification of *Buchnera* in adult aphids prior to larviposition will also include the *Buchnera* present in

their embryos which was estimated at ~36,700 symbionts per embryo for *A. pisum* (Mira and Moran 2002). Similarly quantification of reference genes to estimate aphid tissue density will also include embryonic tissue. The number of embryos produced within parthenogenetic aphids has been shown to be a function of aphid size and influenced by environmental variables (Dixon 1973; Leather and Wellings 1981; Dixon 1987) and differences in ovariole development can cause problems for reference genes to estimate aphid size (Wilkinson *et al.* 2007). Some studies have normalised against aphid fresh weight rather than using reference genes (Wilkinson *et al.* 2007; Chandler *et al.* 2008) although this approach assumes consistent DNA extraction efficiency between samples. By selecting aphids of equal size, at a similar reproductive stage, and maintained in identical culture conditions, the effect of environmental variables on ovariole development was minimized. Thus the comparative Ct method using aphid reference genes and *Buchnera GroEL* was an accurate method for relative comparison of *Buchnera* density in two aphid species, and variation in the data is likely to reflect genotypic differences rather than environmental effects on ovariole production.

5.4.3 The comparative Ct method was sufficient for comparing Buchnera *density.*

The variation in the relative density of *Buchnera* across pea aphid lines confirms the results of Wilkinson and Douglas (1998). Other studies have quantified the amount of *Buchnera* in terms of the number of bacteria per mg of aphid fresh weight (Baumann *et al.* 1995), which is not directly comparable to relative quantification of the *Buchnera GroEL* gene copy number used here. Since *Buchnera* is remarkably polyploid, varying from tens to hundreds of genomes per cell (Komaki and Ishikawa 1999; 2000), gene copy number can only indicate the number of genomes and not the absolute number of bacterial cells. Previous studies such as Wilkinson *et al.* (2007) have used the *Buchnera GroEL* gene to measure changes in *Buchnera* infection density with consistent results and applied an absolute quantification method. The comparative Ct method was less time consuming and less vulnerable to pipetting errors than the absolute quantification method that relies on the generation of standard curves based on known concentrations of plasmid. Given the wide variation in *Buchnera* genome copy number per cell, it is debatable how much extra information would be gained from an absolute quantification method therefore relative

quantification was the method of choice for examining between and within aphid species differences in *Buchnera* density.

5.4.4 Further applications for the SYBR® assay designed for this study.

The real-time qPCR assay designed for this study provided reproducible data for quantification of *Buchnera* in two different aphid species that supported the results of previous studies, showing clonal differences in *Buchnera* in pea aphid (Wilkinson and Douglas 1998) and a reduced density of *Buchnera* when the secondary symbiont *Serratia symbiotica* was present (Koga *et al.* 2003; Sakurai *et al.* 2005; Koga *et al.* 2007). Now developed and optimised, this assay could be applied to address two further questions concerning the function of the *Buchnera* symbiosis. Firstly, the real-time PCR assay could be extended to include *S. symbiotica* as a tool to understand the dynamics of the interaction between *S. symbiotica* and *Buchnera* in pea aphid. Secondly, the assay could be used to address the impact of other ecological factors on the *Buchnera* symbiosis, such as attack by parasitoid wasps. To date, the impact of parasitism on the *Buchnera* symbiosis in pea aphids has been investigated using microscopy techniques (Cloutier and Douglas 2003). The qPCR technique could be applied to examine the relative quantity of *Buchnera* at each stage of parasitoid development from initial oviposition up until the point of mummification, when it becomes impossible to extract DNA from the aphid. To date there has been no thorough investigation of the impact of parasitism on the primary symbiosis in any aphid species other than the pea aphid, so using the SYBR® assay for this purpose would provide new insight into a multitrophic interaction between the parasitoid, aphid and bacteria.

5.4.5 Conclusions

The aim of the work described in this chapter was to investigate the infection density of *Buchnera* using molecular techniques in two different aphid species with varying secondary infections. The infection density of endosymbionts is a key factor in symbiosis function, thus the results of this study shed light on a number of aspects of the ecology of cabbage and pea aphids. Two key conclusions were ascertained by the study. Firstly, the results showed that secondary infection by *S. symbiotica* in the pea aphid could influence

the relative quantity of *Buchnera*, a finding that supported previous studies (Koga *et al.* 2003; Sakurai *et al.* 2005; Koga *et al.* 2007). This is ecologically significant for a number of reasons particularly since it is known that *S. symbiotica* can improve aphid fitness by conferring resistance to parasitoid wasps (Oliver *et al.* 2005; 2008) and increasing thermal tolerance (Montllor *et al.* 2002). There is much speculation as to why seemingly beneficial symbionts such as *S. symbiotica* are not 'fixed' within aphid populations and the possibility that they interfere with the primary symbiosis could be one way that they incur a fitness cost. Antibiotic curing could be used to artificially manipulate the symbiosis and investigate the effect of *S. symbiotica* on the density of *Buchnera* and any knock-on effects on aphid fitness it may incur in the absence of the effect of aphid genotype.

Secondly, clonal patterns in *Buchnera* density in the cabbage aphid did not relate to clonal patterns in infection with either group of accessory bacteria, indicating no direct link between the primary endosymbiont and secondary bacteria infection in the cabbage aphid. This was not entirely unexpected given that the secondary bacteria of cabbage aphids were present at very low densities and probably reside in the aphid gut rather than the haemocoel. Alternative factors contributing to variation in *Buchnera* density could include collection host plant species or other aspects of their ecology including tolerance to plant defensive compounds, such as glucosinolates which are high in brassicas and sequestered by cabbage aphid. In addition, the density of *Buchnera* was greater in the cabbage aphid than the pea aphid, indicating there may be aspects of cabbage aphid biology relating to *Buchnera* infection that are as yet not fully understood. The cabbage aphid–*Buchnera* symbiosis has not been studied in any depth and the results from this study suggest further investigation would be merited. The importance of understanding the dynamics of symbiont infection in different aphid species is highlighted by this study. To further understand symbiont infection the qPCR assay may be applied to investigate the effect of additional trophic levels, such as parasitism, on the primary symbiont in both aphid species giving it a useful practical application in future work.

6. Conclusions and Future Perspectives

6.1 Conclusions and future directions of this study.

6.1.1 Evidence for bacteria mediated fitness trade-offs in cabbage aphid.

It was speculated at the outset of this study that the unique ecology of the cabbage aphid might lead to variation in bacterial complement compared to other previously characterised aphid species. The secondary symbionts previously characterised in the pea aphid (*A. pisum*), *R. insecticola*, *S. symbiotica* and *H. defensa*, were not detected in any of the cabbage aphid lines tested in this study and investigation of the *GroEL* gene in the primary symbiont *Buchnera* revealed a greater relative quantity of gene copies in cabbage aphid relative to pea aphid. In addition, there was a wide diversity of bacteria associated with the cabbage aphid, which could be split into two groups, *Pseudomonas* and *Erwinia* type bacteria. These two groups of bacteria were associated with direct and indirect effects on aphid fitness. The *Pseudomonas* type bacteria appeared to increase aphid fitness while the *Erwinia* type had the opposite effect and was associated with an increase in the fitness of emergent parasitoids. It is possible that the parasitoids preferentially target aphids infected with *Erwinia* type bacteria and this could be a focus of future work. Consequently, infection with the *Erwinia* type bacteria is likely to be unstable and will be lost over time, while the *Pseudomonas* type might be maintained in the aphid. However, the facultative benefits of harbouring secondary bacteria are determined by the environment and infections with one or more bacterial symbiont might not be maintained if harbouring them is costly (Chen *et al.* 2000; Oliver *et al.* 2006). Therefore, infection with *Erwinia* might be beneficial in other circumstances i.e. on other host plants, while harbouring the *Pseudomonas* type bacteria might be costly in some conditions. In western flower thrips, infection with *Erwinia* species has either costs or benefits dependent on the diet of the insect (de Vries *et al.* 2004). Additionally, it has been reported that the phenotypic characteristics of cabbage aphid populations and population demographic parameters vary according to the *Brassica* species with which they are associated (Ruiz-Montoya *et al.* 2005; Ulusoy and Bayhan 2006), which could conceivably relate to bacterial association. Examining host plant related

fitness variability in cabbage aphid infected with *Erwinia* could therefore be a direction of future work.

Infection with secondary bacteria is not ubiquitous throughout aphid populations and taxa suggesting that the beneficial fitness traits they confer are related to environmental pressures such as temperature, host plant interactions and parasitism, and that there are significant fitness trade-offs that occur as a result of harbouring the bacteria (Russell and Moran 2006). Co-evolutionary dynamics between the bacteria and host and vulnerability to selective pressures vary according to the mode of transmission which in the case of the bacteria characterised in cabbage aphid is likely to be predomiantly horizontal rarther than vertical. To determine the stability of the association between the cabbage aphid and the bacteria, and to strengthen the case for a bacteria mediated fitness trade-off, the relative complement of bacteria should be assessed over a longer period of time and on a range of different host plants and environmental conditions. In addition, a number of other ecological aspects of aphid–bacteria interactions should also be addressed to make the study applicable at an arable system scale.

6.1.2 Eliminating the effect of genotype on aphid fitness.

The influence of aphid genotype on symbiont fitness consequences is increasingly recognised as a factor in the outcome of the aphid–endosymbiont interaction (Koga *et al.* 2007). For example, even in the presence of *H. defensa,* variation between aphid clones in their resistance to parasitism is evident (Ferrari *et al.* 2001; 2007). In a test of the relative importance of the aphid genetic background as opposed to the symbiont infection, Oliver *et al.* (2005) introduced different *H. defensa* isolates by microinjection into a common aphid genetic background, and monitored resistance to *A. ervi.* The results showed that multiple *H. defensa* isolates could confer resistance to *A. ervi* irrespective of genetic background of the *A. pisum* clone into which they were injected, suggesting that the symbiont determined the level of resistance and not the aphid genotype. A similar method could be used to eliminate the effect of genotype on the fitness effects associated with infection with the bacteria found in cabbage aphid (e.g. Koga *et al.* 2003). Differential susceptibility to infection with *Erwinia chrysanthemi* across pea aphid clones has been documented (Grenier *et al.* 1994) indicating that differences in relative infection across cabbage aphid

lines could be due to genetic variation in susceptibility to infection. Consequently, an important aspect of future work will be to investigate the interaction between aphid genotype and the fitness consequences associated with harbouring both groups of bacteria. Aphid genetic effects have also been shown to influence host plant specialisation (Leonardo 2004) and super-parasitism success (Vorburger *et al.* 2010b) and are therefore an important factor in shaping aphid population dynamics. In addition, it has been demonstrated that different symbiont genotypes, even closely related isolates, vary in their effect on aphid hosts (Russell and Moran 2006), therefore the genotype of the bacteria might also be important in the interaction.

6.1.3 Expanding the study to a landscape scale.

Understanding the fitness effects of infection with secondary bacteria on aphid populations is important for a number of reasons. As a dynamic component of crop systems aphids interact with their environment on a number of trophic levels each of which could be influenced by their bacterial complement. Aphids are major vectors of several economically important plant pathogens and so any interaction between the bacterial complement of the aphid vector and pathogen transmission has potential knock-on effects on the health and functioning of the arable system. Vector competence depends on genetic factors that govern physiological and molecular interactions between the insect and pathogen, and insect behavioural factors that influence interactions with the host plant and environment. However, despite the numerous examples of work suggesting that bacterial complement can influence various aphid fitness traits, with positive benefits (Montllor *et al.* 2002; Oliver *et al.* 2003; Ferrari *et al.* 2004), or negative effects (Grenier *et al.* 2006; Harada and Ishikawa 1997), there is no research to date on the potentially significant impact of bacteria aphid–symbiont–parasitoid dynamics in spatially extended heterogeneous systems such as large expanses of crop land. The topography of the landscape can influence both aphid and parasitoid populations (Bahlai *et al.* 2010). Roschewitz *et al.* (2005) examined parasitism of cereal aphids in various different landscape types including 'complex' landscapes with hedgerows and field margins and 'simple' landscapes that had high percentages of arable land. They found that complex landscapes had higher parasitism rates (parasitism rate (%) = mummies/(aphids + mummies)*100) on average than simple landscapes, which was

presumably due to greater provision of over-wintering sites, alternative hosts and nectar sources for parasitoids that are provided by hedgerows and field margins. However, they also found that aphid population densities were higher in complex landscapes, possibly resulting from the high availability of winter hosts for holocyclic aphid species.

Aphid population dynamics will ultimately shape parasitism rates in the environment. Brodeur and Rosenheim (2000) investigated the functional response of parasitoid populations to aphid density and found that where aphids were present in large numbers competition both between and within parasitoid species was high. The spatial structure of the landscape can cause variation in the distance between host populations influencing both parasitoid colonisation rate and the potential for local extinction if habitats become particularly fragmented (Rauch and Weisser 2007). However, parasitoids can take advantage of aphid flight for dispersal, as numerous parasitised *M. persicae* alates established new colonies in different areas before being killed by the parasitoid (Feng *et al.* 2007). The dynamics of aphid and parasitoid populations are so closely linked that fluctuations in aphid numbers, both of winged and wingless morphs, will be reflected in parasitoid populations. In addition, when susceptibility to infection with pathogenic *Erwinia* bacteria and wing polymorphism were investigated, it was found that young winged pea aphids were least susceptible to infection (Grenier *et al.* 2006). In arable systems morphological characteristics could therefore influence the dispersal potential of pathogenic bacteria and parasitoids between aphid populations in fragmented landscapes. The effect of landscape heterogeneity on the aphid–parasitoid interaction poses numerous interesting questions, in particular relating to the abundance of natural vegetation, which acts as a refuge for both parasitoids and aphids and could influence the population dynamics of both insects. In addition, the spatially density-dependent nature of insect parasitism would, through a partial refuge effect (i.e. where a proportion of hosts escape parasitism; Pareja *et al.* 2008), diminish selection in favour of infection with facultative bacteria because of the absence of parasitism-induced stimulation of bacterial proliferation.

6.1.4 Transmission of plant pathogens by aphids.

Symbiotic bacteria are important factors in the transmission of persistant viruses. Non-persistent viruses (CaMV) are found in the epidermal cells and phloem sap of the plant

while persistent viruses (BWYV) are acquired predominantly from the phloem sap (Perring *et al.* 1999). In the case of CaMV and TuMV, for which cabbage aphid is the principal vector, the virus particles do not pass across the vector cell membranes (Pirone and Blanc 1996; Gray and Banerjee 1999). They are instead carried externally on the cuticle lining of the vector's stylets where attachment and retention of viral particles is mediated by a non-structural viral protein called a 'helper component' (Pirone and Blanc 1996; Gray and Banerjee 1999). Circulative viruses, such as BWYV, on the other hand are acquired more slowly from the phloem sap and pass into the aphid gut where they reproduce. They then have to pass from the aphid gut to the salivary glands through the aphid haemolymph where they are protected from degradation by a chaperonin protein called SymL, which is synthesised by *Buchnera* (Filichkin *et al.* 1997). Recent studies have suggested that the endosymbiont complement of aphids can be manipulated to potentially inhibit acquisition of persistent viruses. For example, a lectin that can bind to symbiont-derived chaperonins in the aphid gut was found to reduce survival of mustard aphid (*Lipaphis erysimi*) on Indian mustard plants (*Brassica juncea*) when it was expressed ectopically (Banerjee *et al.* 2004; Dutta *et al.* 2005). In addition, availability of the complete genome sequence for *Buchnera* (Shigenobu *et al.* 2000; Pérez-Brocal *et al.* 2006) could allow use of RNA interference to suppress critical proteins such as the SymL chaperonin to limit the transmission of aphid borne viruses. Consequently, by characterising secondary bacteria in aphids as in this study, we can increase our understanding of what is a truly multi-trophic relationship.

Furthermore, cabbage aphid could be a potential vector not only for plant viruses but also plant bacterial pathogens. The cabbage aphid was found to harbour *Erwinia* species in this study and several members of this bacterial genus are economically important plant pathogens. Other insects are vectors for *Erwinia* species that are pathogenic to plants. The cucumber beetles *Acalymma vittatum* and *Diabrotica undecipunctata howardi* (Coleoptera: Chrysomelidae) can vector *Erwinia tracheiphilia* as they travel from flower to flower of wild cucumber plants (*Cucurbita pepo*) collecting pollen (Sasu *et al.* 2010), and the corn flea beetle *Chaetocnema pulicaria* (Melsheimer) (Coleoptera: Chrysomelidae) is the primary vector of *Pantoea (Erwinia) stewartii* (Menelas *et al.* 2006). Cabbage aphid might also, therefore, be able to vector *Erwinia* species as it makes multiple feeding probes and moves from plant to plant. However, there are two potential barriers to transmission of

Erwinia by cabbage aphid that make it unlikely that cabbage aphid is a vector for *Erwinia*. Other studies reported greater bacterial infection levels with *Erwinia* bacteria in later aphid developmental stages, which they attributed to mechanical filtering (Grenier *et al.* 1994; Grenier *et al.* 2006). The stylet of first instar larvae is only 0.6 μm in diameter while *Erwinia* bacteria are 0.5–1 μm in external diameter and 1–3 μm in length, therefore, the bacteria simply might not fit into the stylet of younger aphids (Grenier *et al.* 2006). Also, *Erwinia* infection can lead to high levels of mortality in aphids (Grenier *et al.* 2006) and vectored pathogens that kill their host are uncommon (Goddard 2000). The rapid infection process and anatomy of aphid mouthparts indicate that it would be almost impossible for the bacteria to be inoculated back to the plant via the stylet canals (Grenier *et al.* 1994; Grenier *et al.* 2006) in the same way that persistent viruses are transmitted. To conclusively prove that cabbage aphid does not vector *Erwinia* a future study could use a DAPI (4'-6-diamidino-2-phenylindole**)** fluorescent stain to trace the transmission of live bacterial cells. Given the problems associated with brassica diseases such as soft rot disease, for which *Erwinia* species are responsible, an approach to rule out cabbage aphid as a vector would be of benefit from a disease control perspective. The same technique could also be applied to investigate transmission of *Pseudomonas* bacteria that cause spear rot and *Xanthomonas* bacteria that cause black rot in brassicas, both of which were found associated with cabbage aphid in this study.

6.1.5 Applicability of the study.

At present control of cabbage aphid is largely achieved by pesticide application, so an investigation into the efficiency of *D. rapae* as a biocontrol agent could lead to reductions in pesticide usage. The interaction between cabbage aphid and its parasitoid *D. rapae* should be addressed in the context of the whole crop system taking into account bacterial complement and environmental variables that have been shown to affect the dynamics of the aphid and parasitoid populations. The host specificity of *D. rapae* sub-populations to geographically distinct cabbage aphid populations should also be investigated as high levels of host specificity may also influence the aphid–parasitoid interaction. In addition, the real-time qPCR assay designed in this study could be used to detect whether the parasitoid *D. rapae* is a possible infection route of the bacteria

harboured by the cabbage aphid, highlighting a possible mode of horizontal transmission of bacteria in aphid populations.

Statistical and mathematical models could be fitted to data from large scale aphid fitness and parasitism experiments to determine if values projected by existing parasitism models fit the cabbage aphid–*D. rapae* system. A number of variables, such as bacterial infection status, that previously were not considered as aphid fitness parameters can now be incorporated into the models to improve the fit (e.g. Parry *et al.* 2006; Stark and Acheampong 2007; Bahlai *et al.* 2010). For example, a comparison of the suitability of *M. persicae* and *B. brassicae* as hosts for *D. rapae* based on demographic parameters indicated *D. rapae* was a better biocontrol agent for *M. persicae* than *B. brassicae* (Stark and Acheampong 2007) and including bacterial association in the model could improve our understanding of this result. Additionally, more extensive parasitism experiments will determine to what extent aphid density influences the parasitoid's foraging efficiency, indicating whether it is exhibiting a 'basic' preference for certain host bacterial complements. The work undertaken in this project could be expanded to shed light on a number of other aspects of aphid biology that determine their impact as crop pests. The study also formed a sound basis from which to investigate virus transmission by cabbage aphid lines with different bacterial complements. Addressing the gaps in our knowledge of the impact of bacterial association on aphid–virus interactions and subsequent virus spread would provide a practical and useful tool for agricultural sustainability.

6.2 Future perspectives in aphid–symbiont research.

6.2.1 Potential mechanisms for the establishment of bacterial symbiosis in aphids. **(This section is reproduced from the author's published work Clark *et al.* (2010))**

Presence of bacterial types other than *Buchnera* in cabbage aphid raised the question of whether the bacteria associated with the cabbage aphid had formed a symbiotic relationship with their host. To establish this, it would be necessary to use a microscopy technique, such as FISH, or use the real-time qPCR assay on carefully dissected out tissues, to determine both the localisation of the bacteria and the fidelity of vertical transmission. Although the likely location for the bacteria detected in this study was the gut, it is possible

that we are observing the early stages of the formation of a symbiosis. There are examples of gut microbiota that represent all aspects of microbial relationships ranging from pathogens to obligate mutualists (Dillon and Dillon 2004). The evolutionary dynamics of symbiosis from a free living to a vertically transmitted lifestyle leads to a reduction in bacterial genome size that has profound consequences for the life history strategy of both the bacteria and the insect host. Obligate primary endosymbionts contribute to the nutrition of the insect host and are essential for the survival of the insect, while secondary facultative bacteria have more diverse effects on host fitness and are not essential for host insect survival. Consequently, facultative and obligate symbionts differ significantly in the mechanisms required for transmission and maintenance in populations of herbivorous insects. Primary obligate symbionts have become integrated into the anatomy and metabolism of the host insect while facultative bacteria must invade and overcome insect physiological defences and mechanical barriers to establish an infection (Moran *et al.* 2008). It has been shown that following experimental horizontal infection by microinjection low transmission efficiency and negative fitness effects can act as a barrier to the establishment of new symbioses, although some 'facultative' symbionts are able to overcome these obstacles and persist within aphid populations (Russell and Moran 2005). The mechanisms that allow bacteria to gain entry to their insect host, and the extent to which they trigger or evade insect immune responses, is likely to be a significant focus of future research as data emerges on the molecular processes that might be involved.

Type 3 secretion systems (T3SSs) used by invading pathogenic bacteria are thought to also be employed by most of the insect facultative symbionts in the Enterobacteriaceae (Dale *et al.* 2002; Dale and Moran 2006). Five distinct secretion systems (1 to 5) are present in Gram-negative bacteria, where they facilitate the translocation of macromolecules across inner and outer bacterial cell membranes into the periplasmic or extracellular space or to the surface of the bacterial cell (Pugsley *et al.* 2004). In general, gene clusters encoding T3SSs are present in the genomes of recently established microbial endosymbionts of insects (Dale *et al.* 2002; Dale and Moran 2006) such as *H. defensa* (Degnan *et al.* 2009), *R. insecticola* (Degnan *et al.* 2010) and *Arsenophonus* species (Dale *et al.* 2006). Obligate symbionts lack genes encoding T3SSs, with the exception of the weevil primary endosymbionts SOPE (*Sitophilus oryzae* primary endosymbiont) and SZPE

(*Sitophilus zeamais* primary endosymbiont) (Dale and Moran 2006). Weevil SOPE and SPZE symbionts along with the close relative tsetse fly (Diptera: Glossinidae) symbiont *Sodalis glossinidus*, exhibit T3SSs with a common ancestor indicating that horizontal transmission may have facilitated infection of the weevil host by a *Sodalis*-type symbiont (Dale *et al.* 2002). Although SOPE and SZPE are primary symbionts with T3SSs their symbiotic origin is relatively recent (Lefèvre *et al.* 2004) indicating that T3SSs may be necessary for the initial establishment of symbiotic bacteria in host tissues. The absence of T3SSs from the genome of primary endosymbionts such as *Wigglesworthia*, *Blochmannia* and *Buchnera* might indicate that these genes have been lost as a consequence of genome reduction (Dale and Moran 2006), or that alternative invasion strategies have been employed during the evolution of ancient symbioses. Interestingly, the redundant flagellar apparatus genes in *Buchnera*, which show differential expression during embryo and adult stages of aphid development, are suggested to function as protein-secreting transporters analogous to T3SSs (Bermingham *et al.* 2009). In addition, urease is a virulence factor in several bacterial and fungal pathogens and by analogy, the urease gene cluster in the genome sequence of the obligate endosymbiont *Blochmannia* might represent a remnant of a former pathogenicity factor that has been involved in symbiosis establishment (Gil *et al.* 2003). A number of mechanisms exist by which the bacteria found in cabbage aphid could become established and form a symbiotic relationship with their host. Members of the *Erwinia* genus, for example, that were found in cabbage aphid possess a T3SS (Toth *et al.* 2003).

6.2.2 Interactions between bacteria and the aphid immune system. (This section is reproduced from the author's published work Clark *et al.* (2010))

Following host invasion, bacterial symbionts must attain sufficient infection levels for vertical transmission without simultaneously compromising the survival of the host by challenging the host immune system. Insects exhibit a variety of immune defences including clotting, production of antimicrobial substances and phagocytosis (Gillespie and Kanost 1997). Bacteria that survive the insect's constitutive immune response are subsequently more resistant to it (Haine *et al.* 2008). Consequently, the interaction between the insect immune system and their symbiotic bacteria is likely to be highly co-ordinated.

Aphids depend on their symbiotic bacteria for survival but also require an active immune system to protect against infection by parasitoids and pathogens. However, recent whole genome sequencing has revealed that several of the genes that are critical for recognition, signalling and destruction of infecting microbes are absent in the pea aphid (Gerardo *et al.* 2010). Aphids share some defence systems with other insects including heat shock proteins and the Toll and Jak/STAT signalling pathway but several central genes are missing including defensins and peptidoglycan recognition proteins (PGRPs) (Gerardo *et al.* 2010). In other insects, production of antimicrobial peptides and up-regulation of other genes involved in the immune response is common following bacterial infection but aphids do not exhibit these responses suggesting that they possess significantly reduced or altered immune systems (Altincicek *et al.* 2008).

The aphid immune response to challenges from microbial pathogens is limited largely due to the absence of genes central to insect immune function (Gerardo *et al.* 2010) and therefore they should be highly vulnerable to infection by bacteria such as *Erwinia*. High vulnerability to infection by pathogens is not however a stable evolutionary strategy and there is a significant amount of speculation as to why the aphid immune system is so drastically reduced. There are costly fitness trade-offs associated with maintaining immunity against bacterial pathogens as has been demonstrated in *Pseudomonas aeruginosa* infection in *Drosophila* (Ye *et al.* 2009), therefore the balance between investment in defence versus reproduction is critical. Current opinion suggests that the pressure for rapid colonisation and reproduction coupled with the fitness trade-offs between insect defence against natural enemies and aphid fecundity and survival (Gwynn *et al.* 2005) has selected for fast reproduction over high levels of immunity in aphids (Godfray 2010). However, several alternative hypotheses have also been proposed to explain reduced immunity to microbial infection in aphids. The first hypothesis suggests that aphids feeding on sterile phloem sap rarely encounter pathogens and therefore do not require a robust immune system (Altincicek *et al.* 2008). However, since aphids can acquire bacteria from the plant surfaces or from the honeydew of infected aphids (Stavrinides *et al.* 2009) this hypothesis seems the least likely. Following bacterial infection, it has been reported that aphids undergo an increase in reproduction and a second hypothesis speculates that this 'terminal reproduction' might represent a strategy for maximizing offspring survival

(Altincicek *et al.* 2008). After infection with fungal pathogens, however, aphid reproduction is reduced (Scarborough *et al.* 2005) indicating that terminal reproduction is not a general strategy to compensate for a poor immune response in aphids. Additionally, it has been speculated that the symbiont-mediated host protection provided by extracellular facultative symbionts (Fukatsu *et al.* 2000) explains why aphids have minimal or specialised antimicrobial defences (Altincicek *et al.* 2008) as the cost of expression of immune genes is outweighed by the fitness benefits associated with harbouring secondary symbionts (Gerardo *et al.* 2010). However, it is not clear whether the presence of secondary facultative bacteria in aphids is a cause or a consequence of reduced antimicrobial defences. Indeed, presence of symbiotic bacteria could increase susceptibility of aphids to other bacterial pathogens (Grenier *et al.* 2006).

Applying post-genomic tools to compare symbiotic and non-symbiotic insect and bacteria species would be a productive area for future research to reveal the insect and bacterial molecular mechanisms involved in maintaining a functional endosymbiosis. Sequencing of the *Buchnera* genome, for example, has revealed a lack of genes for cell surface components, suggesting that *Buchnera* is structurally vulnerable to attack compared to other free-living bacteria that possess surface structures designed to evade the host immune system (Shigenobu *et al.* 2000). The structural fragility of *Buchnera* could be a consequence of a prolonged intracellular lifestyle that provides protection both from the host immune system and from pathogen attack (Shigenobu *et al.* 2000). The secondary symbiont *H. defensa* has considerably more cell surface structural genes than *Buchnera* indicating that it is better suited to survival in the fluctuating conditions that are likely to be associated with the extracellular phases of its life style (Degnan *et al.* 2009), as is also the case for the cabbage aphid *Pseudomonas* and *Erwinia* type bacteria. Thus, the interaction between the host immune system and the adaptive physiology of facultative bacteria could be essential for the establishment and maintenance of symbiosis.

6.3 Concluding remarks

For facultative bacteria to become established in the host insect population they must exert a positive selection pressure leading to an increase in the frequency of the symbiont-containing host phenotype (Moran *et al.* 2008). The positive benefit of

harbouring facultative bacteria when selection pressures are high will be balanced by the energetic cost of maintaining the symbiosis (Gwynn *et al.* 2005). This aspect of symbiosis research, known as ecological immunology, distinguishes between the long-term evolutionary costs of possessing symbiotic bacteria that provide fitness benefits and the short-term fitness costs of the association (Gwynn *et al.* 2005). Positive selection for insect fitness traits conferred by secondary facultative symbiotic bacteria could eventually drive the insect–microbe association towards an obligate symbiosis. This study found that cabbage aphid harbours bacteria, of a different type to pea aphid, that might also influence aphid phenotypic complexity but differ in their mode of transmission and vulnerability to selective pressures. However, more work is needed to establish the localisation of the bacteria within the insect, determine the mode of transmission, investigate the role of insect and bacterial genetic variation and to extend the study to a landscape scale before the full scope of the work is realised. Relatively little is known about the physiological and metabolic aspects of the aphid–parasitoid–symbiont interaction. However, recent sequencing of the whole genome of three species of parasitoid wasp in the *Nasonia* genus (Hymenoptera: Pteromalidae: Pteromalinae) (The Nasonia Genome Working Group 2010) and the pea aphid (The International Aphid Genomics Consortium 2010) has provided researchers with an extremely useful resource for investigating gene expression in insect–parasitoid–symbiont interactions (Godfray 2010; Pennisi 2010). As a consequence, proteomic techniques, which have already been employed to study aphid resistance and susceptibility responses to parasitism (Nguyen *et al.* 2008) are likely to be used more widely along with additional systems level approaches (e.g. Thomas *et al.* 2009).

Aphid–bacterial association should be viewed as a complex and dynamic process involving multiple players that can have a profound influence on aphid phenotypic complexity (Moran 2007) and their interaction with other organisms and the abiotic environment (reviewed in Clark *et al.* 2010). The results from this study highlight the extent to which bacteria are key players in shaping the ecology of aphids by influencing their fitness and interaction with other trophic groups, including natural enemies, pathogens and microbial symbionts. As a consequence of this work we now have more information regarding the ecology of cabbage aphid in arable systems, which will provide the basis for future studies to improve control of cabbage aphid in Scotland.

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Appendix 1: Derivation of the formula $2^{-\Delta\Delta C_T}$

The relative quantity of target (16S) normalised to an endogenous control (*Ef1-α*) and relative to a calibrator was calculated using the formula:

Relative Quantity (RQ) = $2^{-\Delta\Delta C_T}$

Derivation (Applied Biosystems Real-Time PCR Systems Chemistry Guide 2004)

The equation that describes the exponential amplification of PCR is:

$X_n = X_o \times (1 + E_X)^n$

where:

X_n = number of target molecules at cycle n
X_o = initial number of target molecules
E_X = efficiency of target amplification
n = number of cycles

The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$X_T = X_o \times (1 + E_X)^{C_{T,X}} = K_X$

where:

X_T = threshold number of target molecules
$C_{T,X}$ = threshold cycle for target amplification
K_X = constant

A similar equation for the endogenous control reaction is:

$R_T = R_o \times (1 + E_R)^{C_{T,R}} = K_R$

where:

R_T = threshold number of reference molecules
R_o = initial number of reference molecules
E_R = efficiency of reference amplification
$C_{T,R}$ = threshold cycle for reference amplification
K_R = constant

Dividing X_T by R_T gives the expression:

$X_T / R_T = X_o \times (1+ E_X)^{C_{T,X}} / R_o \times (1+E_R)^{C_{T,R}} = K_X / K_R = K$

The exact values of X_T and R_T depend on a number of factors, including:

• Reporter dye used in the probe
• Sequence context effects on the fluorescence properties of the probe
• Efficiency of probe cleavage
• Purity of the probe
• Setting of the fluorescence threshold.

Therefore, the constant K does not have to be equal to 1.

Assuming efficiencies of the target and the reference are the same:

$E_X = E_R = E$

$X_o/R_o \times (1+E)^{C_{T,X}-C_{T,R}} = K$

OR

$X_N \times (1+E)^{\Delta C_T} = K$

where:

$X_N = X_o/R_o$, the normalised amount of target
$\Delta C_T = C_{T,X} - C_{T,R}$, the difference in threshold cycles for target and reference

Rearranging gives the expression:

$X_N = K\,(1+E)^{-\Delta C_T}$

The final step is to divide the X_N for any sample (q) by the X_N for the calibrator (cb):

$X_{N,q}/\,X_{N,cb} = K \times (1+E)^{-\Delta C_{T,q}} / \,K \times (1+E)^{-\Delta C_{T,cb}} = (1+E)^{-\Delta\Delta C_T}$

Where:

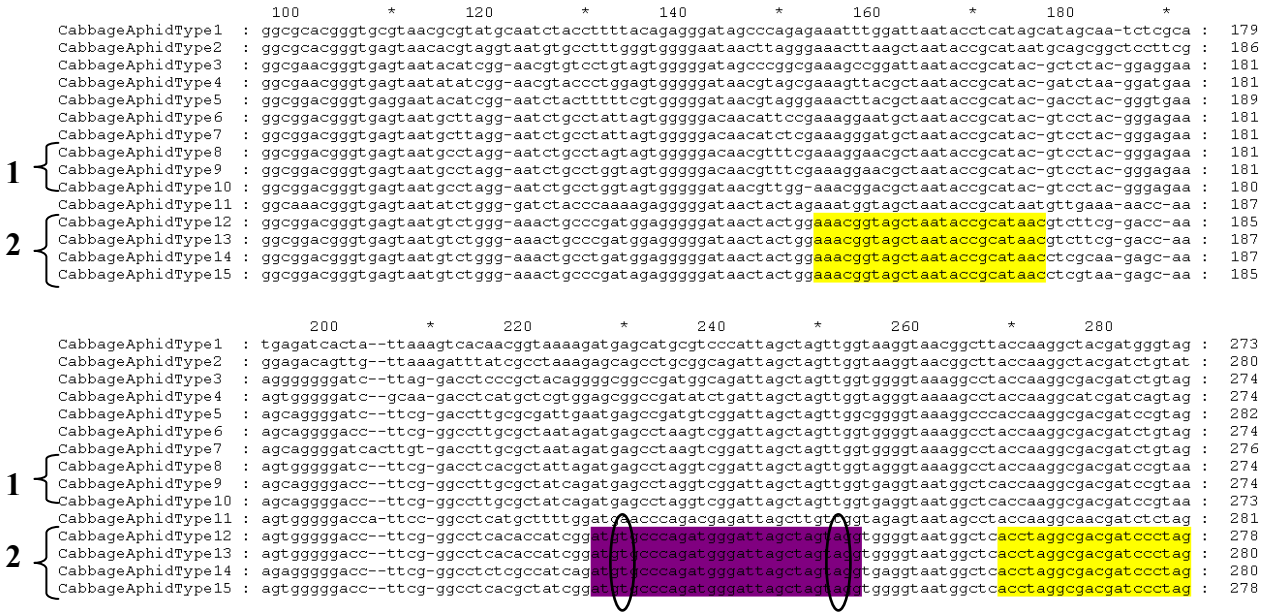
$\Delta\Delta C_T = \Delta C_{T,q} - \Delta C_{T,cb}$

The amplicons were designed and optimised according to the Applied Biosystems Assay Design Guidelines (required amplicon size < 150 bp) (Applied Biosystems Real-time PCR Systems Chemistry Guide), so the efficiency is close to 1. Therefore, the amount of target, normalised to an endogenous control and relative to a calibrator, is given by:

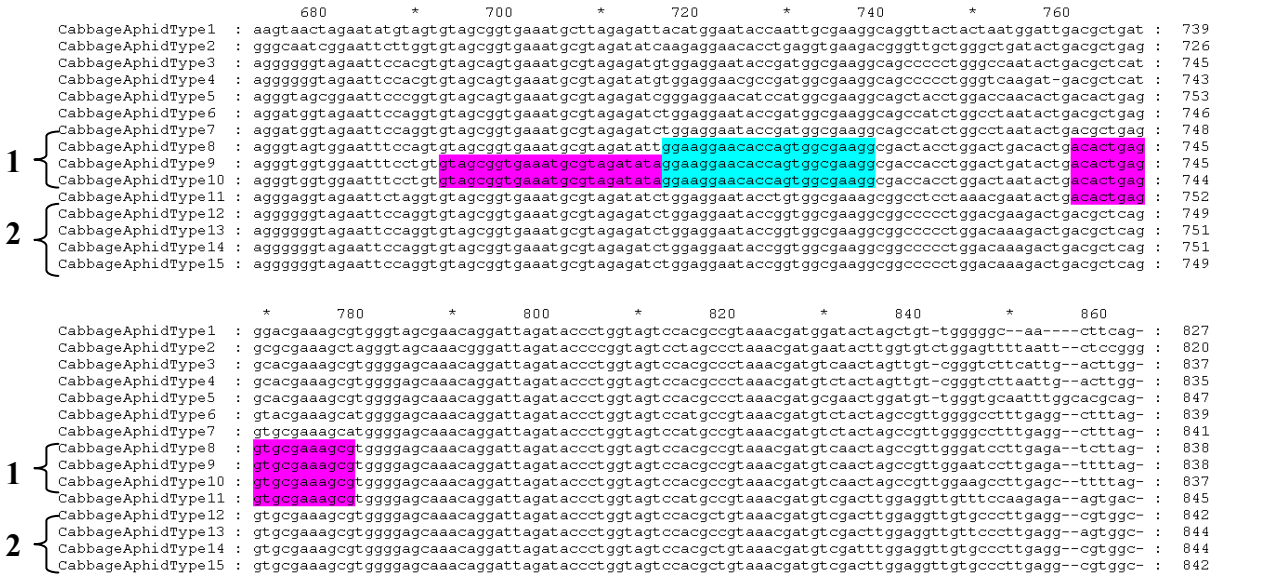
$2^{-\Delta\Delta C_T}$

Appendix 2: Alignments illustrating primer and probe sites for Group 1 and Group 2 bacteria and the endogenous reference gene *Ef1-α*.

16S consensus sequence from all fifteen different cabbage sequence types aligned to illustrate the high level of sequence conservation between sequence types. Primers sites for CABAC1-155F and CABAC1-269R = yellow and probe site for CABAC1-228P = purple. The two unique nucleotide bases that determine the specificity of the CABAC1-228P Taqman® probe for Group 2 bacteria are circled.



16S consensus sequence from all fifteen different cabbage sequence types aligned to illustrate the specificity of probe CABAC2-718P (light blue) for Group 1 bacteria (types 8, 9 and 10). Primers CABAC2-695F and CABAC2-762R are highlighted in pink.



Aphid elongation factor 1-α (*Ef1-α*) sequence from multiple aphid species aligned to illustrate the position of primers ApisEF1-422F and ApisEF1-537R (light green) and probe ApisEF1-506P (dark green) in cabbage aphid. Accession numbers from the NCBI database precede each sequence except those which were generated in this study which, are preceded by a *

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                                    *       460         *       480         *       500         *       520         *
EU358931L.pseudobrassicae : ctctggatggaatggagacaacatgttggaagtttccga--------aaagatgtc-ttggttcaaaggatggactgttgaa-cgtaaa :  477
EU358928B.brassicae       : ctctggatggaatggagacaacatgttggaagtttccga--------aaagatgtc-ttggttcaaaggatggaatgttgaa-cgtaaa :  477
AY219734B.brassicae       : ctctggatggaatggagacaacatgttggaagtttccga--------aaagatgtc-ttggttcaaaggatggaatgttgaa-cgtaaa :  510
*ELC0605B.brassicae       : ----------------------------------------ggatcgaaaga-gtc-ttggttcaa-ggatggaatgttgaa-cgtaaa :   44
*ELC0606B.brassicae       : ----------------------------------------ggatcgaa-ga-gtc-t-ggttcaa-ggatggaatgttgaa-cgtaaa :   42
*ELC0607B.brassicae       : ----------------------------------------tggatcgaa-ga-gtcctggttcaa-ggatggaatgttgaa-cgtaaa :   45
*JF99A.pisum              : -------------------------------------tttgatcgaa-aatgtcgtgggttcag-ggatgggctgttgaaacgtaaa :   48
FM174698A.pisum           : ctctggatggaatggagacaacatgttggaagtttccga--------aaaaatgtcgt-ggttcaagggatgggctgttgaa-cgtaaa :  500
AY219737A.pisum           : ctctggatggaatggagacaacatgttggaagtttccga--------aaaaatgtcgt-ggttcaagggatgggccgttgaa-cgtaaa :  499
EU358933M.persicae        : ctctggatggaatggagacaacatgttggaagtttccga--------aaagatgtc-atggttcaaaggatgggccgttgaa-cgtaaa :  466
EU358908A.fabae           : ctctggatggaatggagacaacatgttggaagtttccga--------caaaatgac-atggttcaaaggatggaatgttgaa-cgtaaa :  471
EU358936R.padi            : ctctggatggaatggagacaacatgttggaagtttccga--------caagatgtc-atggttcaaaggatggaatgttgaa-cgtaaa :  474
DQ418870A.grossulariae    : ctctggatggaacggagacaacatgttggaagtttccga--------caaaatgtc-atggttcaaaggatggaatgttgaa-cgtaaa :  282
DQ499608F.formicaria      : ctctggatggaacggagacaacatgttggaagtttctga--------gaaaatgcc-atggttcaagggatggaatgttgaa-cgtaaa :  471
FM174683C.cedri           : ctctggatggaacggtgacaacatgttggaagtttccga--------aaaaatgcc-atggttcaagggatggaatgttgaa-cgtaaa :  505
EU358903A.smilacifoliae   : ctctggatggaatggtgacaacatgttggaagtttccga--------caaaatgac-atggttcaaaggatggaatgttgaa-cgtaaa :  475
AF068465U.rurale          : ctctggatggaatggagacaacttgttggaagtttccga--------aaaaatgtcgt-ggttcaagggatgggccgttgaa-cgtaaa :  501

                                  540         *       560         *       580         *       600         *       620
EU358931L.pseudobrassicae : gaaggaaaggccgacggtaa-a-tg-tttgattgaagctttagacgccatcctaccacccagtcgcccaactgacaaggctctccgtct :  563
EU358928B.brassicae       : gaaggaaaggctgacggtaa-a-tg-tttgattgaagctttagatgctatcctgccacccagtcgcccaactgacaaggctctccgtct :  563
AY219734B.brassicae       : gaaggaaaggctgacggtaa-a-tg-tttgattgaagctttagatgctatcctgccacccagtcgcccaactgacaaggctctccgtct :  596
*ELC0605B.brassicae       : gaaggaaaggccgacggtcaca-tg-atggaatggag---------------------------------------------------- :   79
*ELC0606B.brassicae       : gaaggaaaggccgacggtct-t-t--tcggaacttccaacatgttgtctccattccatccag-------------------------- :  100
*ELC0607B.brassicae       : gaaggaaaggccgacggTCactctg-atggaatggag---------------------------------------------------- :   81
*JF99A.pisum              : gaaggaaaggccgacggctc-t-gg-atggaatggag---------------------------------------------------- :   82
FM174698A.pisum           : gaaggaaaggccgacggtaa-a-tg-tttgattgaagctttagacgctatcctgccacccagtcgcccaactgacaaggctctccgtct :  586
AY219737A.pisum           : gaaggaaaggctgacggtaa-a-tg-tttgattgaagctttagacgctatcctgccacccagccgcccaactgacaaggctctccgtct :  585
EU358933M.persicae        : gaaggaaaggctgatggtaa-a-tg-tttgattgaagctttagacgctatcctgccacccagtcgcccaactgacaaggctctccgtct :  552
EU358908A.fabae           : gaaggaaaggctgacggcaa-a-tg-tctgattgaagctttagacgctatcctacccccagtcgcccaactgacaaggctctccgtct :  557
EU358936R.padi            : gaaggaaaggctgatggtaa-a-tg-tctgattgaagctttagacgctatcctacccccagtcgcccaactgacaaggctcttcgtct :  560
DQ418870A.grossulariae    : gaaggaaaggccgacggtaa-a-tg-tctgattgaagctttagacgctatcctacccccagtcgcccaactgacaaggctctccgtct :  368
DQ499608F.formicaria      : gaaggaaaggccgacggcaa-a-tg-tttgatcgaagctttagatgctatcctgccacccagtcgcccaactgacaaggctcttcgact :  557
FM174683C.cedri           : gaaggaaaggctgacggtaa-a-tg-tttaattgaagctttggatgctatcctgccacccagtcgtccaactgacaaagctctccgtct :  591
EU358903A.smilacifoliae   : gaaggaaaggccgacggtaa-a-tg-tctgattgaagctttagacgctatcctacccccagtcgaccaactgacaaggctctccgtct :  561
AF068465U.rurale          : gaaagaaaggcagatggtaa-a-tg-tttaatnaaaactttanacgctatcctgccacccaatcgcccaactgaaaaaggtcttcgtct :  587
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Appendix 3: Relative Quantification of Group 1 and Group 2 bacteria using the ΔΔCt method.

Appendix 3.1: Relative Quantification of Group 1 *Pseudomonas* type cabbage aphid bacteria using the ΔΔCt method.

	Average Ct						$16S_{Normalised}$	[c]Range $16S_{Normalised}$	
Aphid Line	**Target 16S**	**Reference *Ef1-α***	[a]**Average ΔCt**	**ΔCtvab**	**-ΔΔCt**	[b]**ΔΔCt**	**RQ $2^{-(\Delta\Delta Ct)}$**	**Min**	**Max**
ELC0604P Calibrator	43.72	24.86	18.86	0.728	0	0	1	0.37	2.69
ELC0604P	43.72	24.86	18.86	0.728	0	0	1	0.37	2.69
ELC0619	34.68	24.72	9.96	0.252	8.9	-8.9	477.71	339.22	672.75
ELC0606P	35.98	25.19	10.79	0.583	8.07	-8.07	268.73	121.71	593.33
ELC0613	33.22	25.02	8.2	0.405	10.66	-10.66	1618.00	933.30	2805.03
ELC0611	34.83	24.02	10.81	0.756	8.05	-8.05	265.03	94.89	740.19
ELC0618	30.49	24.91	5.58	0.203	13.28	-13.28	9946.68	7549.26	13105.46
ELC0605	28.9	24.67	4.23	0.151	14.63	-14.63	25355.30	20652.66	31128.75
ELC0701	39.2	24.8	14.4	0.247	4.46	-4.46	22.01	15.73	30.78
ELC0610	40.25	24.21	16.04	0.537	2.82	-2.82	7.06	3.40	14.65
ELC0703	37.76	24.52	13.24	0.581	5.62	-5.62	49.18	22.34	108.29
ELC0803	37.94	24.23	13.71	0.502	5.15	-5.15	35.51	17.95	70.23
ELC0612	36.69	25.2	11.49	1.287	7.37	-7.37	165.42	28.79	950.48
ELC0801	33.7	24.62	9.08	0.304	9.78	-9.78	879.17	581.71	1328.74
ELC0606	31.16	25.58	5.58	0.579	13.28	-13.28	9946.68	4529.58	21842.34
ELC0607	33.35	25.79	7.56	0.223	11.3	-11.3	2521.38	1862.36	3413.60
ELC0604	27.18	24.62	2.56	0.069	16.3	-16.3	80684.28	73464.52	88613.57

Appendix 3.2: Relative Quantification of Group 2 *Erwinia* type cabbage aphid bacteria using the ΔΔCt method.

	Average Ct						$16S_{Normalised}$	[c]Range $16S_{Normalised}$	
Aphid Line	**Target 16S**	**Reference *Ef1-α***	[a]**Average ΔCt**	**ΔCtvab**	**-ΔΔCt**	[b]**ΔΔCt**	**RQ $2^{-(\Delta\Delta Ct)}$**	**Min**	**Max**
ELC0703 Calibrator	34.22	24.19	10.03	1.01	0	0	1	0.25	3.93
ELC0604P	34.05	24.8	9.25	0.10	0.78	-0.78	1.72	1.50	1.97
ELC0619	23.03	24.52	-1.49	0.13	11.52	-11.52	2936.74	2478.07	3480.31
ELC0606P	31.95	25.15	6.8	0.33	3.23	-3.23	9.38	6.03	14.59
ELC0613	32.56	24.87	7.69	0.38	2.34	-2.34	5.06	3.03	8.47
ELC0611	28.55	23.83	4.72	0.43	5.31	-5.31	39.67	22.09	71.25
ELC0618	27.06	24.7	2.36	0.26	7.67	-7.67	203.66	143.25	289.54
ELC0605	27.54	24.07	3.47	0.26	6.56	-6.56	94.35	65.92	135.06
ELC0701	30.19	24.51	5.68	0.31	4.35	-4.35	20.39	13.44	30.95
ELC0610	33.11	24.08	9.03	0.02	1	-1	2	1.96	2.04
ELC0703	34.22	24.19	10.03	1.01	0	0	1	0.25	3.93
ELC0803	33.26	24.13	9.13	0.25	0.9	-0.9	1.87	1.33	2.62
ELC0612	32.21	25.23	6.98	0.45	3.05	-3.05	8.28	4.48	15.30
ELC0801	34.02	24.46	9.56	0.14	0.47	-0.47	1.39	1.15	1.68
ELC0606	30.91	25.58	5.33	0.27	4.7	-4.7	25.99	18.11	37.31
ELC0607	30.59	25.79	4.8	0.51	5.23	-5.23	37.53	18.87	74.63
ELC0604	21.49	24.5	-3.01	0.02	13.04	-13.04	8422.31	8230.02	8619.09

a. The ΔC_T value is determined by subtracting the average *Ef1-α* C_T value from the average 16S C_T value.
For example, ΔC_T ELC0703 = 34.22 -24.19 = 10.03.
The standard deviation of the difference is calculated from the standard deviations of the *Ef1-α* and 16S values to give ΔC_Tvab.
b. The calculation of $-\Delta\ \Delta C_T$ involves subtracting ΔC_T calibrator value from the ΔC_T target value.
For example, $-\Delta\ \Delta C_T$ ELC0619 = -(ΔC_T ELC0619 - ΔC_T ELC0703) = -(-1.49 – 10.03) = 11.52
This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value.
c. The range given for $16S_{Normalised}$ relative to the calibrator (ELC0703) is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$
with $\Delta\Delta C_T$ + s and $\Delta\Delta C_T$ – s, where s = the standard deviation of the $\Delta\Delta C_T$ value.
For example, Range ELC0619 has a $\Delta\Delta C_T$ value of -11.25 and a range of Standard deviation = 0.13. Therefore: $2^{-\Delta\Delta C_T} = 2^{11.25}$ = 2936.74. The lower range limit = $2^{11.25+0.13}$ = 2478.07. The upper range limit = $2^{11.25-0.13}$ = 3480.31.

Appendix 4: Interspecific Relative Quantification of *Buchnera GroEL* using the ΔΔCt method.

Appendix 4.1: Relative Quantification of *Buchnera GroEL* in pea aphid relative to cabbage aphid.

Relative Quantification of *Buchnera GroEL* in pea aphid relative to cabbage aphid using β-tubulin as the endogenous reference.

	Average Ct						$GroEL_{Normalised}$	[c]Range $GroEL_{Normalised}$	
Aphid Line	Target *GroEL*	Reference β-tubulin	[a]Average ΔCt	ΔCtvab	-ΔΔCt	[b]ΔΔCt	RQ $2^{-(\Delta\Delta Ct)}$	Min	Max
ELC0610 Calibrator	21.15	27.75	-6.6	0.336	0	0	1.000	0.634	1.579
LL01	19.9	25.64	-5.74	1.251	-0.86	0.86	0.551	0.101	3.015
JF99	20.28	25.54	-5.26	1.355	-1.34	1.34	0.395	0.063	2.489
JF98	19.83	24.45	-4.62	1.314	-1.98	1.98	0.253	0.043	1.511
PS01	20.6	24.95	-4.35	0.549	-2.25	2.25	0.210	0.100	0.443
TLW03/01	19.65	24.92	-5.27	0.558	-1.33	1.33	0.398	0.186	0.849
JF01	20.14	25.12	-4.98	1.4	-1.62	1.62	0.325	0.049	2.180
SH3	20.14	24.74	-4.6	0.375	-2	2	0.250	0.150	0.416
N116	19.61	24.65	-5.04	0.233	-1.56	1.56	0.339	0.247	0.465
N127	19.78	24.69	-4.91	0.386	-1.69	1.69	0.310	0.183	0.524

Relative Quantification of *Buchnera GroEL* in pea aphid relative to cabbage aphid using *Ef1-α* as the endogenous reference.

	Average Ct						$GroEL_{Normalised}$	[c]Range $GroEL_{Normalised}$	
Aphid Line	Target *GroEL*	Reference *Ef1-α*	[a]Average ΔCt	ΔCtvab	-ΔΔCt	[b]ΔΔCt	RQ $2^{-(\Delta\Delta Ct)}$	Min	Max
ELC0610 Calibrator	20.67	25.72	-5.05	0.42	0	0	1.000	0.565	1.769
LL01	19.85	24.4	-4.55	1.245	-0.5	0.5	0.707	0.130	3.838
JF99	20.06	24.44	-4.38	1.341	-0.67	0.67	0.629	0.102	3.886
JF98	19.63	23.64	-4.01	1.377	-1.04	1.04	0.486	0.075	3.158
PS01	20.48	24.12	-3.64	0.386	-1.41	1.41	0.376	0.223	0.636
TLW03/01	19.59	24.25	-4.66	0.444	-0.39	0.39	0.763	0.417	1.395
JF01	19.95	24.15	-4.2	1.433	-0.85	0.85	0.555	0.079	3.887
SH3	20.14	24	-3.86	0.283	-1.19	1.19	0.438	0.298	0.644
N116	19.61	23.86	-4.25	0.332	-0.8	0.8	0.574	0.366	0.902
N127	19.78	23.89	-4.11	0.301	-0.94	0.94	0.521	0.346	0.785

* See Appendix 3 for explanation of a, b and c.

Appendix 4.2: Relative Quantification of *Buchnera GroEL* in cabbage aphid relative to pea aphid.

Relative Quantification of *Buchnera GroEL* in cabbage aphid relative to pea aphid using β-tubulin as the endogenous reference.

	Average Ct						$GroEL_{Normalised}$	[c]Range $GroEL_{Normalised}$	
Aphid Line	Target *GroEL*	Reference β-tubulin	[a]Average ΔCt	ΔCtvab	-ΔΔCt	[b]ΔΔCt	RQ $2^{-(\Delta\Delta Ct)}$	Min	Max
LL01 Calibrator	19.9	25.64	-5.74	1.251	0	0	1.000	0.183	5.472
ELC0605	21.55	28.22	-6.67	0.7	0.93	-0.93	1.905	0.736	4.931
ELC0606	21.17	28.06	-6.89	0.336	1.15	-1.15	2.219	1.406	3.503
ELC0607	21.63	28.2	-6.57	0.378	0.83	-0.83	1.778	1.064	2.971
ELC0604	20.74	27.58	-6.84	0.693	1.1	-1.1	2.144	0.836	5.496
ELC0610	21.15	27.75	-6.6	0.492	0.86	-0.86	1.815	0.930	3.541
ELC0701	20.84	27.65	-6.81	0.76	1.07	-1.07	2.099	0.748	5.895
ELC0611	20.21	26.56	-6.35	0.564	0.61	-0.61	1.526	0.709	3.284
ELC0619	20.14	26.32	-6.18	0.208	0.44	-0.44	1.357	1.023	1.800
ELC0703	21.94	27.49	-5.55	0.878	-0.19	0.19	0.877	0.266	2.890

Relative Quantification of *Buchnera GroEL* in cabbage aphid relative to pea aphid using *Ef1-α* as the endogenous reference.

	Average Ct						$GroEL_{Normalised}$	[c]Range $GroEL_{Normalised}$	
Aphid Line	Target *GroEL*	Reference *Ef1-α*	*[a]Average ΔCt	ΔCtvab	-ΔΔCt	[b]ΔΔCt	RQ $2^{-(\Delta\Delta Ct)}$	Min	Max
LL01 Calibrator	19.85	24.4	-4.55	1.245	0	0	1.000	0.184	5.427
ELC0605	20.89	26.03	-5.14	0.588	0.59	-0.59	1.505	0.677	3.346
ELC0606	20.98	26.3	-5.32	0.42	0.77	-0.77	1.705	0.964	3.017
ELC0607	21.45	26.14	-4.69	0.582	0.14	-0.14	1.102	0.500	2.430
ELC0604	20.72	26.03	-5.31	0.511	0.76	-0.76	1.693	0.846	3.391
ELC0610	20.67	25.72	-5.05	0.513	0.5	-0.5	1.414	0.704	2.839
ELC0701	20.61	25.77	-5.16	1.02	0.61	-0.61	1.526	0.382	6.102
ELC0611	20.21	25.31	-5.1	0.537	0.55	-0.55	1.464	0.706	3.037
ELC0619	20.14	25.07	-4.93	0.346	0.38	-0.38	1.301	0.813	2.082
ELC0703	21.95	26.4	-4.45	0.782	-0.1	0.1	0.933	0.322	2.700

Appendix 5: Intraspecific Relative Quantification of *Buchnera GroEL* using the ΔΔCt method.

Appendix 5.1: Relative Quantification of *Buchnera GroEL* across pea aphid lines.

Relative Quantification of *Buchnera GroEL* across pea aphid lines using β-tubulin as the endogenous reference.

	Average Ct						*GroEL* Normalised	[c]Range *GroEL* Normalised	
Aphid Line	**Target *GroEL***	**Reference β-tubulin**	**[a]Average ΔCt**	**ΔCtvab**	**-ΔΔCt**	**[b]ΔΔCt**	**RQ $2^{-(\Delta\Delta Ct)}$**	**Min**	**Max**
LL01 Calibrator	19.9	25.64	-5.74	1.251	0	0	1.000	0.183	5.472
LL01	19.9	25.64	-5.74	1.251	0	0	1.000	0.183	5.472
JF99	20.28	25.54	-5.26	1.355	-0.48	0.48	0.717	0.114	4.518
JF98	19.83	24.45	-4.62	1.314	-1.12	1.12	0.460	0.077	2.742
PS01	20.6	24.95	-4.35	0.549	-1.39	1.39	0.382	0.181	0.804
TLW03/01	19.65	24.92	-5.27	0.558	-0.47	0.47	0.722	0.338	1.541
JF01	20.14	25.12	-4.98	1.4	-0.76	0.76	0.590	0.088	3.956
SH3	20.14	24.74	-4.6	0.375	-1.14	1.14	0.454	0.273	0.755
N116	19.61	24.65	-5.04	0.233	-0.7	0.7	0.616	0.449	0.845
N127	19.78	24.69	-4.91	0.386	-0.83	0.83	0.563	0.333	0.950

Relative Quantification of *Buchnera GroEL* across pea aphid lines using *Ef1-α* as the endogenous reference.

	Average Ct						*GroEL* Normalised	[c]Range *GroEL* Normalised	
Aphid Line	**Target *GroEL***	**Reference *Ef1-α***	**[a]Average ΔCt**	**ΔCtvab**	**-ΔΔCt**	**[b]ΔΔCt**	**RQ $2^{-(\Delta\Delta Ct)}$**	**Min**	**Max**
LL01 Calibrator	19.85	24.4	-4.55	1.245	0	0	1.000	0.184	5.427
LL01	19.85	24.4	-4.55	1.245	0	0	1.000	0.184	5.427
JF99	20.06	24.44	-4.38	1.341	-0.17	0.17	0.889	0.144	5.496
JF98	19.63	23.64	-4.01	1.377	-0.54	0.54	0.688	0.106	4.466
PS01	20.48	24.12	-3.64	0.386	-0.91	0.91	0.523	0.315	0.899
TLW03/01	19.59	24.25	-4.66	0.444	-0.11	0.11	1.079	0.507	1.694
JF01	19.95	24.15	-4.2	1.433	-0.35	0.35	0.785	0.112	5.497
SH3	20.14	24	-3.86	0.283	-0.69	0.69	0.620	0.422	0.910
N116	19.61	23.86	-4.25	0.332	-0.3	0.3	0.812	0.517	1.275
N127	19.78	23.89	-4.11	0.301	-0.44	0.44	0.737	0.490	1.110

Appendix 5.2: Relative Quantification of *Buchnera GroEL* across cabbage aphid lines.

Relative Quantification of *Buchnera GroEL* across cabbage aphid lines using β-tubulin as the endogenous reference.

	Average Ct						$GroEL_{Normalised}$	[c]Range $GroEL_{Normalised}$	
Aphid Line	**Target *GroEL***	**Reference β-tubulin**	***[a]Average ΔCt**	**ΔCtvab**	**-ΔΔCt**	**[b]ΔΔCt**	**RQ $2^{-(\Delta\Delta Ct)}$**	**Min**	**Max**
ELC0703 Calibrator	21.94	27.49	-5.55	0.878	0	0	1.000	0.303	3.296
ELC0605	21.55	28.22	-6.67	0.7	1.12	-1.12	2.173	0.840	5.626
ELC0606	21.17	28.06	-6.89	0.336	1.34	-1.34	2.532	1.604	3.996
ELC0607	21.63	28.2	-6.57	0.378	1.02	-1.02	2.028	1.213	3.389
ELC0604	20.74	27.58	-6.84	0.693	1.29	-1.29	2.445	0.954	6.269
ELC0610	21.15	27.75	-6.6	0.492	1.05	-1.05	2.071	1.061	4.040
ELC0701	20.84	27.65	-6.81	0.76	1.26	-1.26	2.395	0.853	6.725
ELC0611	20.21	26.56	-6.35	0.564	0.8	-0.8	1.741	0.809	3.746
ELC0619	20.14	26.32	-6.18	0.208	0.63	-0.63	1.548	1.167	2.053
ELC0703	21.94	27.49	-5.55	0.878	0	0	1.000	0.303	3.296

Relative Quantification of *Buchnera GroEL* across cabbage aphid lines using *Ef1-α* as the endogenous reference.

	Average Ct						$GroEL_{Normalised}$	[c]Range $GroEL_{Normalised}$	
Aphid Line	**Target *GroEL***	**Reference *Ef1-α***	**[a]Average ΔCt**	**ΔCtvab**	**-ΔΔCt**	**[b]ΔΔCt**	**RQ $2^{-(\Delta\Delta Ct)}$**	**Min**	**Max**
ELC0703 Calibrator	21.95	26.4	-4.45	0.782	0	0	1.000	0.346	2.893
ELC0605	20.89	26.03	-5.14	0.588	0.69	-0.69	1.613	0.726	3.586
ELC0606	20.98	26.3	-5.32	0.42	0.87	-0.87	1.828	1.033	3.234
ELC0607	21.45	26.14	-4.69	0.582	0.24	-0.24	1.181	0.536	2.604
ELC0604	20.72	26.03	-5.31	0.511	0.86	-0.86	1.815	0.907	3.634
ELC0610	20.67	25.72	-5.05	0.513	0.6	-0.6	1.516	0.755	3.043
ELC0701	20.61	25.77	-5.16	1.02	0.71	-0.71	1.636	0.409	6.540
ELC0611	20.21	25.31	-5.1	0.537	0.65	-0.65	1.569	0.757	3.255
ELC0619	20.14	25.07	-4.93	0.346	0.48	-0.48	1.395	0.872	2.232
ELC0703	21.95	26.4	-4.45	0.782	0	0	1.000	0.346	2.893

* See Appendix 3 for explanation of a, b and c.